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POLYPEPTIDE VARIANTS WITH ALTERED EFFECTOR FUNCTION**BACKGROUND OF THE INVENTION**

10 This is a continuation-in-part application claiming priority to non-provisional application No. 09/483,588, filed January 14, 2000, which claims priority under 35 USC § 119 to provisional application No. 60/116,023 filed January 15, 1999, the entire disclosures of which are incorporated herein by reference.

Field of the Invention

15 The present invention concerns polypeptides comprising a variant Fc region. More particularly, the present invention concerns Fc region-containing polypeptides that have altered effector function as a consequence of one or more amino acid modifications in the Fc region thereof.

Description of Related Art

20 Antibodies are proteins which exhibit binding specificity to a specific antigen. Native antibodies are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (V_H) followed by a number of constant domains. Each light chain has a variable domain at one end (V_L) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains.

30 The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are responsible for the binding specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) both in the light chain and the heavy chain variable domains. The more highly conserved portions of the variable domains are called the framework regions (FRs). The variable domains of native heavy and light chains each comprise four FRs,

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largely adopting a β -sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the β -sheet structure. The CDRs in each chain are held together in close proximity by the FRs and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)).

The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector functions. Depending on the amino acid sequence of the constant region of their heavy chains, antibodies or immunoglobulins can be assigned to different classes.

There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), *e.g.* IgG1, IgG2, IgG3, and IgG4; IgA1 and IgA2. The heavy chain constant regions that correspond to the different classes of immunoglobulins are called α , δ , ϵ , γ , and μ , respectively. Of the various human immunoglobulin classes, only human IgG1, IgG2, IgG3 and IgM are known to activate complement; and human IgG1 and IgG3 mediate ADCC more effectively than IgG2 and IgG4.

A schematic representation of the native IgG1 structure is shown in Fig. 1, where the various portions of the native antibody molecule are indicated. Papain digestion of antibodies produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. The crystal structure of the human IgG Fc region has been determined (Deisenhofer, *Biochemistry* 20:2361-2370 (1981)). In human IgG molecules, the Fc region is generated by papain cleavage N-terminal to Cys 226. The Fc region is central to the effector functions of antibodies.

The effector functions mediated by the antibody Fc region can be divided into two categories: (1) effector functions that operate after the binding of antibody to an antigen (these functions involve the participation of the complement cascade or Fc receptor (FcR)-bearing cells); and (2) effector functions that operate independently of antigen binding (these functions confer persistence in the circulation and the ability to be transferred across cellular barriers by transcytosis). Ward and Ghetie, *Therapeutic Immunology* 2:77-94 (1995).

While binding of an antibody to the requisite antigen has a neutralizing effect that might prevent the binding of a foreign antigen to its endogenous target (*e.g.* receptor or ligand), binding alone may not remove the foreign antigen. To be efficient in removing and/or destructing foreign antigens, an antibody should be endowed with both high affinity binding to its antigen, and efficient effector functions.

Fc receptor (FcR) binding

The interaction of antibodies and antibody-antigen complexes with cells of the immune system effects a variety of responses, including antibody-dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) (reviewed in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997); Ward and Ghetie, *Therapeutic Immunol.* 2:77-94 (1995); as well as Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991)).

Several antibody effector functions are mediated by Fc receptors (FcRs), which bind the Fc region of an antibody. FcRs are defined by their specificity for immunoglobulin isotypes; Fc receptors for IgG antibodies are referred to as Fc γ R, for IgE as Fc ϵ R, for IgA as Fc α R and so on. Three subclasses of Fc γ R have been identified: Fc γ RI (CD64), Fc γ RII (CD32) and Fc γ RIII (CD16). Because each Fc γ R subclass is encoded by two or three genes, and alternative RNA splicing leads to multiple transcripts, a broad diversity in Fc γ R isoforms exists. The three genes encoding the Fc γ RI subclass (Fc γ RIA, Fc γ RIB and Fc γ RIC) are clustered in region 1q21.1 of the long arm of chromosome 1; the genes encoding Fc γ RII isoforms (Fc γ RIIA, Fc γ RIIB and Fc γ RIIC) and the two genes encoding Fc γ RIII (Fc γ RIIIA and Fc γ RIIIB) are all clustered in region 1q22. These different FcR subtypes are expressed on different cell types (reviewed in Ravetch and Kinet, *Annu. Rev. Immunol.* 9:457-492 (1991)). For example, in humans, Fc γ RIIIB is found only on neutrophils, whereas Fc γ RIIIA is found on macrophages, monocytes, natural killer (NK) cells, and a subpopulation of T-cells. Notably, Fc γ RIIIA is the only FcR present on NK cells, one of the cell types implicated in ADCC.

Fc γ RI, Fc γ RII and Fc γ RIII are immunoglobulin superfamily (IgSF) receptors; Fc γ RI has three IgSF domains in its extracellular domain, while Fc γ RII and Fc γ RIII have only two IgSF domains in their extracellular domains.

Another type of Fc receptor is the neonatal Fc receptor (FcRn). FcRn is structurally similar to major histocompatibility complex (MHC) and consists of an α -chain noncovalently bound to β 2-microglobulin.

The binding site on human and murine antibodies for Fc γ R have been previously mapped to the so-called "lower hinge region" consisting of residues 233-239 (EU index numbering as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD. (1991)). Woof *et al.* *Molec. Immunol.* 23:319-330 (1986); Duncan *et al.* *Nature* 332:563 (1988); Canfield and Morrison, *J. Exp. Med.* 173:1483-1491 (1991); Chappel *et al.*, *Proc. Natl. Acad. Sci USA* 88:9036-9040 (1991). Of residues 233-239, P238 and S239 have been cited as possibly being involved in binding, but these two residues have never been evaluated by substitution or deletion.

Other previously cited areas possibly involved in binding to Fc γ R are: G316-K338

(human IgG) for human Fc γ RI (by sequence comparison only; no substitution mutants were evaluated) (Woof *et al. Molec. Immunol.* 23:319-330 (1986)); K274-R301 (human IgG1) for human Fc γ RIII (based on peptides) (Sarmay *et al. Molec. Immunol.* 21:43-51 (1984)); Y407-R416 (human IgG) for human Fc γ RIII (based on peptides) (Gergely *et al. Biochem. Soc. Trans.* 12:739-743 (1984)); as well as N297 and E318 (murine IgG2b) for murine Fc γ RII (Lund *et al., Molec. Immunol.*, 29:53-59 (1992)).

Pro331 in IgG3 was changed to Ser, and the affinity of this variant to target cells analyzed. The affinity was found to be six-fold lower than that of unmutated IgG3, indicating the involvement of Pro331 in Fc γ RI binding. Morrison *et al., Immunologist*, 2:119-124 (1994); and Canfield and Morrison, *J. Exp. Med.* 173:1483-91 (1991).

C1q binding

C1q and two serine proteases, C1r and C1s, form the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway. C1q is a hexavalent molecule with a molecular weight of approximately 460,000 and a structure likened to a bouquet of tulips in which six collagenous "stalks" are connected to six globular head regions. Burton and Woof, *Advances in Immunol.* 51:1-84 (1992). To activate the complement cascade, it is necessary for C1q to bind to at least two molecules of IgG1, IgG2, or IgG3 (the consensus is that IgG4 does not activate complement), but only one molecule of IgM, attached to the antigenic target. Ward and Ghetie, *Therapeutic Immunology* 2:77-94 (1995) at page 80.

Based upon the results of chemical modifications and crystallographic studies, Burton *et al. (Nature*, 288:338-344 (1980)) proposed that the binding site for the complement subcomponent C1q on IgG involves the last two (C-terminal) β -strands of the CH2 domain. Burton later suggested (*Molec. Immunol.*, 22(3):161-206 (1985)) that the region comprising amino acid residues 318 to 337 might be involved in complement fixation.

Duncan and Winter (*Nature* 332:738-40 (1988)), using site directed mutagenesis, reported that Glu318, Lys320 and Lys322 form the binding site to C1q. The data of Duncan and Winter were generated by testing the binding of a mouse IgG2b isotype to guinea pig C1q. The role of Glu318, Lys320 and Lys322 residues in the binding of C1q was confirmed by the ability of a short synthetic peptide containing these residues to inhibit complement mediated lysis. Similar results are disclosed in U.S. Patent No. 5,648,260 issued on July 15, 1997, and U.S. Patent No. 5,624,821 issued on April 29, 1997.

The residue Pro331 has been implicated in C1q binding by analysis of the ability of human IgG subclasses to carry out complement mediated cell lysis. Mutation of Ser331 to Pro331 in IgG4 conferred the ability to activate complement. (Tao *et al., J. Exp. Med.*, 178:661-667 (1993); Brekke *et al., Eur. J. Immunol.*, 24:2542-47 (1994)).

From the comparison of the data of the Winter group, and the Tao *et al.* and Brekke *et al.* papers, Ward and Ghetie concluded in their review article that there are at least two different regions involved in the binding of C1q: one on the β -strand of the CH2 domain bearing the Glu318, Lys320 and Lys322 residues, and the other on a turn located in close proximity to the same β -strand, and containing a key amino acid residue at position 331.

Other reports suggested that human IgG1 residues Leu235, and Gly237, located in the lower hinge region, play a critical role in complement fixation and activation. Xu *et al.*, *Immunol.* 150:152A (Abstract) (1993). WO94/29351 published December 22, 1994 reports that amino acid residues necessary for C1q and FcR binding of human IgG1 are located in the N-terminal region of the CH2 domain, *i.e.* residues 231 to 238.

It has further been proposed that the ability of IgG to bind C1q and activate the complement cascade also depends on the presence, absence, or modification of the carbohydrate moiety positioned between the two CH2 domains (which is normally anchored at Asn297). Ward and Ghetie, *Therapeutic Immunology* 2:77-94 (1995) at page 81.

SUMMARY OF THE INVENTION

The present invention provides a variant of a parent polypeptide comprising an Fc region, which variant mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more effectively, or binds an Fc gamma receptor (Fc γ R) with better affinity, than the parent polypeptide and comprises at least one amino acid modification in the Fc region. The polypeptide variant may, for example, comprise an antibody or an immunoadhesin. The Fc region of the parent polypeptide preferably comprises a human Fc region; *e.g.*, a human IgG1, IgG2, IgG3 or IgG4 Fc region. The polypeptide variant preferably comprises an amino acid modification (*e.g.* a substitution) at any one or more of amino acid positions 256, 290, 298, 312, 326, 330, 333, 334, 360, 378 or 430 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

In addition, the invention provides a polypeptide comprising a variant Fc region with altered Fc gamma receptor (Fc γ R) binding affinity, which polypeptide comprises an amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376, 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. The variant Fc region preferably comprises a variant human IgG Fc region, *e.g.*, a variant human IgG1, IgG2, IgG3 or IgG4 Fc region. In this respect, it is noted that, in the work in the above-cited art where the parent polypeptide had a non-human murine Fc

region, different residues from those identified herein were thought to impact FcR binding. For example, in the murine IgG2b/murine Fc γ RII system, IgG E318 was found to be important for binding (Lund *et al. Molec. Immunol.* 27(1):53-59 (1992)), whereas E318A had no effect in the human IgG/human Fc γ RII system (Table 6 below).

5 In one embodiment, the polypeptide variant with altered Fc γ R binding activity displays reduced binding to an Fc γ R and comprises an amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 292, 293, 294, 295, 296, 298, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439 of the Fc region, wherein the numbering of the
10 residues in the Fc region is that of the EU index as in Kabat.

For example, the polypeptide variant may display reduced binding to an Fc γ RI and comprise an amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 327 or 329 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

15 The polypeptide variant may display reduced binding to an Fc γ RII and comprise an amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 335, 338, 373, 376, 414, 416, 419, 435, 438 or 439 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

20 The polypeptide variant of interest may display reduced binding to an Fc γ RIII and comprise an amino acid modification at one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 293, 294, 295, 296, 301, 303, 322, 327, 329, 338, 340, 373, 376, 382, 388, 389, 416, 434, 435 or 437 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

25 In another embodiment, the polypeptide variant with altered Fc γ R binding affinity displays improved binding to the Fc γ R and comprises an amino acid modification at any one or more of amino acid positions 255, 256, 258, 267, 268, 272, 276, 280, 283, 285, 286, 290, 298, 301, 305, 307, 309, 312, 315, 320, 322, 326, 330, 331, 333, 334, 337, 340, 360, 378, 398 or 430 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in
30 Kabat.

For example, the polypeptide variant may display increased binding to an Fc γ RIII and, optionally, may further display decreased binding to an Fc γ RII. An exemplary such variant comprises amino acid modification(s) at position(s) 298 and/or 333 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

35 The polypeptide variant may display increased binding to an Fc γ RII and comprise an

amino acid modification at any one or more of amino acid positions 255, 256, 258, 267, 268, 272, 276, 280, 283, 285, 286, 290, 301, 305, 307, 309, 312, 315, 320, 322, 326, 330, 331, 337, 340, 378, 398 or 430 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. Such polypeptide variants with increased binding to an FcγRII may optionally further display decreased binding to an FcγRIII and may, for example, comprise an amino acid modification at any one or more of amino acid positions 268, 272, 298, 301, 322 or 340 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

The invention further provides a polypeptide comprising a variant Fc region with altered neonatal Fc receptor (FcRn) binding affinity, which polypeptide comprises an amino acid modification at any one or more of amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439 or 447 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. Such polypeptide variants with reduced binding to an FcRn may comprise an amino acid modification at any one or more of amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439 or 447 (and preferably one or more of amino acid positions 253, 254, 435 or 436) of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. The above-mentioned polypeptide variants may, alternatively, display increased binding to FcRn and comprise an amino acid modification at any one or more of amino acid positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434 (and preferably one or more of amino acid positions 238, 256, 307, 311, 312, 380, 382 or 434) of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

The polypeptide variant may be in one of the following classes of variants as shown in Table 6 herein (with increased, reduced or unchanged binding to a receptor being calculated as described with respect to that table):

1. A polypeptide variant which displays reduced binding to FcγRI, FcγRIIA, FcγRIIB and FcγRIIIA comprising an amino acid modification at any one or more of amino acid positions 238, 265, 327 or 329 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

2. A polypeptide variant with reduced binding to FcγRII and FcγRIIIA comprising an amino acid modification at any one or more of amino acid positions 270, 295 or 327 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

3. A polypeptide variant which displays increased binding to Fc γ RII and Fc γ RIIIA comprising an amino acid modification at any one or more of amino acid positions 256 or 290 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

4. A polypeptide variant which displays increased binding to Fc γ RII but unchanged binding to Fc γ RIIIA and comprises an amino acid modification at any one or more of amino acid positions 255, 258, 267, 272, 276, 280, 285, 286, 307, 309, 315, 326, 331, 337, 378 or 430 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

5. A polypeptide variant which displays increased binding to Fc γ RII and reduced binding to Fc γ RIIIA comprising an amino acid modification at any one or more of amino acid positions 268, 301 or 322 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

6. A polypeptide variant which displays reduced binding to Fc γ RII but unchanged Fc γ RIIIA binding and comprises an amino acid modification at any one or more of amino acid positions 292 or 414 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

7. A polypeptide variant which displays reduced binding to Fc γ RII and improved binding to Fc γ RIIIA comprising an amino acid modification at amino acid position 298 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

8. A polypeptide variant which displays reduced binding to Fc γ RIIIA but unchanged FcRII binding comprising an amino acid modification at any one or more of amino acid positions 239, 269, 293, 296, 303, 327, 338 or 376 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

9. A polypeptide variant which displays improved binding to Fc γ RIIIA but unchanged FcRII binding comprising an amino acid modification at any one or more of amino acid positions 333 or 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

10. A polypeptide variant which displays altered binding to FcRn but unchanged Fc γ R binding and comprises an amino acid modification at any one or more of amino acid positions 253, 254, 288, 305, 311, 312, 317, 360, 362, 380, 382, 415, 424, 433, 434, 435 or 436 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

The polypeptide variant herein with improved ADCC activity is preferably selected from:

1. A polypeptide variant which comprises an amino acid modification at any one or more

of amino acid positions 298, 333 or 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

2. A polypeptide variant which comprises amino acid modifications at two or more of amino acid positions 298, 333, 334 or 339 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

3. A polypeptide variant which comprises an amino acid modification at amino acid position 298 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

4. A polypeptide variant which comprises amino acid modifications at amino acid positions 298 and 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

5. A polypeptide variant which comprises amino acid modifications at three or more of amino acid positions 298, 333, 334 or 339 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

6. A polypeptide variant which comprises amino acid modifications at amino acid positions 298, 333 and 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

The invention provides a polypeptide variant selected from:

1. A polypeptide comprising a variant Fc region which comprises amino acid modifications at amino acid positions 298, 333 and 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

2. A polypeptide comprising a variant Fc region which comprises amino acid modifications at amino acid positions 298 and 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

3. A polypeptide comprising a variant Fc region which comprises an amino acid modification at amino acid position 298 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

4. A polypeptide comprising a variant Fc region which comprises amino acid modifications at two or all of amino acid positions 307, 380 and 434 of the Fc region, wherein the numbering of the residues is that of the EU index as in Kabat.

In a further embodiment, the invention provides a polypeptide comprising a variant Fc region with altered affinity for an Fc γ R allotype (e.g. an Fc γ R11A-Phe158, Fc γ R11A-Val158, Fc γ R11A-R131 or Fc γ R11A-H131 allotype), which polypeptide comprises an amino acid modification at any one or more of amino acid positions 265, 267, 268, 270, 290, 298, 305, 307, 315, 317, 320, 331, 333 or 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat. Preferably, the polypeptide displays increased

binding to Fc γ R1IIIA-Phe158 and hence has improved therapeutic effectiveness, particularly in patients who express Fc γ R1IIIA-Phe158. Polypeptide variants with increased binding to Fc γ R1IIIA-Phe158 preferably comprise an amino acid modification at any one or more of amino acid positions 290, 298, 333 or 334 of the Fc region, wherein the numbering of the residues in the Fc region is that of the EU index as in Kabat.

The invention also provides a composition comprising the polypeptide variant and a physiologically or pharmaceutically acceptable carrier or diluent. This composition for potential therapeutic use is sterile and may be lyophilized.

Diagnostic and therapeutic uses for the polypeptide variants disclosed herein are contemplated. In one diagnostic application, the invention provides a method for determining the presence of an antigen of interest comprising exposing a sample suspected of containing the antigen to the polypeptide variant and determining binding of the polypeptide variant to the sample. In one therapeutic application, the invention provides a method of treating a mammal suffering from or predisposed to a disease or disorder, comprising administering to the mammal a therapeutically effective amount of a polypeptide variant as disclosed herein, or of a composition comprising the polypeptide variant and a pharmaceutically acceptable carrier.

The invention further provides: isolated nucleic acid encoding the polypeptide variant; a vector comprising the nucleic acid, optionally, operably linked to control sequences recognized by a host cell transformed with the vector; a host cell containing the vector; a method for producing the polypeptide variant comprising culturing this host cell so that the nucleic acid is expressed and, optionally, recovering the polypeptide variant from the host cell culture (e.g. from the host cell culture medium).

The invention further provides a method for making a variant Fc region with altered Fc receptor (FcR) binding affinity, or altered antibody-dependent cell-mediated cytotoxicity (ADCC) activity, comprising:

- (a) introducing one or more amino acid modifications into an Fc region of a parent polypeptide in order to generate a variant Fc region;
- (b) determining binding of the variant Fc region to an FcR, or determining ADCC activity of the variant Fc region.

Step (b) of the method may comprise determining binding of the variant Fc region to one or more FcRs *in vitro*. Moreover, the method may result in the identification of a variant Fc region with improved FcR binding affinity, or with improved ADCC activity, in step (b) thereof. Where step (b) comprises determining binding of the Fc region to an FcR, the FcR may, for example, be human Fc gamma receptor III (Fc γ R1II). Where step (b) comprises determining binding of the variant Fc region to at least two different FcRs, the FcRs tested preferably include human Fc gamma receptor II (Fc γ R1I) and human Fc gamma receptor III (Fc γ R1II).

Brief Description of the Drawings

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of a native IgG. Disulfide bonds are represented by heavy lines between CH1 and CL domains and the two CH2 domains. V is variable domain; C is constant domain; L stands for light chain and H stands for heavy chain.

Figure 2 shows C1q binding of wild type (wt) C2B8 antibody; C2B8 antibody with a human IgG2 constant region (IgG2); and variants K322A, K320A and E318A.

Figure 3 depicts C1q binding of variants P331A, P329A and K322A.

Figures 4A and 4B depict the amino acid sequences of E27 anti-IgE antibody light chain (Fig. 4A; SEQ ID NO:1) and heavy chain (Fig. 4B; SEQ ID NO:2).

Figure 5 is a schematic diagram of the "immune complex" prepared for use in the FcR assay described in Example 1. The hexamer comprising three anti-IgE antibody molecules (the "Fc region-containing polypeptide") and three IgE molecules (the "first target molecule") is shown. IgE has two "binding sites" for the anti-IgE antibody (E27) in the Fc region thereof. Each IgE molecule in the complex is further able to bind two VEGF molecules ("the second target polypeptide"). VEGF has two "binding sites" for IgE.

Figure 6 shows C1q binding results obtained for variants D270K and D270V compared to wild type C2B8.

Figure 7 depicts complement dependent cytotoxicity (CDC) of variants D270K and D270V, compared to wild type C2B8.

Figure 8 shows C1q binding ELISA results for 293 cell-produced wild type C2B8 antibody (293-Wt-C2B8), CHO-produced wild type C2B8 antibody (CHO-Wt-C2B8) and various variant antibodies.

Figure 9 shows C1q binding ELISA results obtained for wild type (wt) C2B8 and various variant antibodies as determined in Example 3.

Figure 10 depicts the three-dimensional structure of a human IgG Fc region, highlighting residues: Asp270, Lys326, Pro329, Pro331, Lys322 and Glu333.

Figure 11 shows C1q binding ELISA results obtained for wild type C2B8 and various variant antibodies as determined in Example 3.

Figure 12 shows C1q binding ELISA results obtained for wild type C2B8 and double variants, K326M-E333S and K326A-E333A.

Figure 13 shows CDC of wild type C2B8 and double variants, K326M-E333S and K326A-E333A.

Figure 14 depicts C1q binding ELISA results obtained for C2B8 with a human IgG4

(IgG4), wild type C2B8 (Wt-C2B8), C2B8 with a human IgG2 constant region (IgG2), and variant antibodies as described in Example 3.

Figures 15A and 15B show binding patterns for parent antibody (E27) to Fc γ RIIB and Fc γ RIIIA. Figure 15A shows the binding pattern for the humanized anti-IgE E27 IgG1 as a monomer (open circles), hexamer (closed squares), and immune complex consisting of multiple hexamers (closed triangles) to a recombinant GST fusion protein of the human Fc γ RIIB (CD32) receptor α subunit. The hexameric complex (closed squares) was formed by the mixture of equal molar concentrations of E27 (which binds to the Fc region of human IgE) and a human myeloma IgE. The hexamer is a stable 1.1 kD complex consisting of 3 IgG molecules (150 kD each) and 3 IgE molecules (200 kD each). The immune complex (closed triangles) was formed sequentially by first mixing equal molar concentrations of E27 and recombinant anti-VEGF IgE (human IgE with Fab variable domains that bind human VEGF) to form the hexamer. Hexamers were then linked to form an immune complex by the addition of 2x molar concentration of human VEGF, a 44 kD homodimer which has two binding sites for the anti-VEGF IgE per mole of VEGF. Figure 15B shows the binding pattern to a recombinant GST fusion protein of the human Fc γ RIIIA (CD16) receptor α subunit.

Figure 16A shows the binding of immune complexes using different antigen-antibody pairs to recombinant GST fusion protein of the Fc γ RIIA receptor α subunit. Figure 16B shows the binding of the same antigen-antibody pairs to the GST fusion protein of the Fc γ RIIIA receptor α subunit. Closed circles represent binding of human IgE:anti-IgE E27 IgG1; open circles represent binding of human VEGF:humanized anti-VEGF IgG1.

Figure 17 summarizes differences in binding selectivity of some alanine variants between the different Fc γ Rs. Binding of alanine variants at residues in the CH2 domain of anti-IgE E27 IgG1 are shown to Fc γ RIIA, Fc γ RIIB, and Fc γ RIIIA. Type 1 abrogates binding to all three receptors: D278A (265 in EU numbering). Type 2 improves binding to Fc γ RIIA and Fc γ RIIB, while binding to Fc γ RIIIA is unaffected: S280A (267 in EU numbering). Type 3 improves binding to Fc γ RIIA and Fc γ RIIB, but reduces binding to Fc γ RIIIA: H281A (268 in EU numbering). Type 4 reduces binding to Fc γ RIIA and Fc γ RIIB, while improving binding to Fc γ RIIIA: S317A (298 in EU numbering). Type 5 improves binding to Fc γ RIIIA, but does not affect binding to Fc γ RIIA and Fc γ RIIB: E352A, K353A (333 and 334 in EU numbering).

Figures 18A and 18B compare the Fc γ RIIIA protein/protein assay and CHO GPI-Fc γ RIIIA cell based assay, respectively. Figure 18A illustrates binding of selected alanine variants to Fc γ RIIIA-GST fusion protein. S317A (298 in EU numbering) and S317A/K353A (298 and 334 in EU numbering) bind better than E27 wildtype, while D278A (265 in EU numbering) almost completely abrogates binding. Figure 18B illustrates that a similar pattern of binding is found on CHO cells expressing a recombinant GPI-linked form of Fc γ RIIIA.

Figures 19A and 19B compare the Fc γ R1IB protein/protein assay and CHO GPI-Fc γ R1IB cell based assay, respectively. Figure 19A illustrates binding of selected alanine variants to Fc γ R1IB-GST fusion protein. H281A (268 in EU numbering) binds better than E27 wildtype while S317A (298 in EU numbering) shows reduced binding. Figure 19B illustrates that a similar pattern of binding is found on CHO cells expressing a recombinant membrane bound form of Fc γ R1IB.

Figure 20 shows single alanine substitutions in the CH2 domain of anti-HER2 IgG1 (HERCEPTIN®) that influence Fc γ R1IIIA binding in both the protein-protein and cell-based assays alter the ability to bind to Fc γ R1IIIA on peripheral blood mononuclear cell (PBMC) effector cells. Recombinant humanized anti-HER2 (HERCEPTIN®), which binds to HER2-expressing SK-BR-3 breast tumor cells, was preincubated with ⁵¹Cr-labeled SK-BR-3 cells for 30 minutes (opsonization) at 100 ng/ml (filled circles) and 1.25 ng/ml (filled squares). Keeping the SK-BR-3 tumor target cell concentration constant, the ratio of effector cells was increased from 0 to 100. The spontaneous cytotoxicity in the absence of antibody (hatched squares) was 20% at an effector:target (E:T) ratio of 100:1. A single alanine mutation that did not affect Fc γ R1IIIA binding, variant G31 = R309A (292 in EU numbering), did not effect ADCC (filled triangles). A single alanine mutation that only slightly increased binding to Fc γ R1IIIA, variant G30 = K307A (290 in EU numbering), also showed slightly improved ADCC (*i.e.*, a 1.1 fold improvement in ADCC activity, calculated as area under the curve) at 1.25 ng/ml at all E:T ratios (filled diamonds) compared to wildtype antibody at 1.25 ng/ml (filled square). A single alanine mutation that decreased binding to Fc γ R1IIIA, variant G34 = Q312A (295 in EU numbering), also showed decreased ADCC activity (filled inverted triangles).

Figure 21 illustrates that a single alanine mutation which had the most improved binding to Fc γ R1IIIA, variant G36 = S317A (298 in EU numbering), in the protein-protein and cell-based assays also showed the most improvement in ADCC (filled triangles) among the variants compared to wildtype (closed squares) at 1.25 ng/ml. G36 displayed a 1.7 fold improvement in ADCC activity, calculated as area under the curve. Variants G17 = E282A (269 in EU numbering) and G18 = D283A (270 in EU numbering) both showed reduced binding to Fc γ R1IIIA as well as reduced efficacy in ADCC. The effector cells were PBMCs.

Figure 22A depicts alignments of native sequence IgG Fc regions. Native sequence human IgG Fc region sequences, humIgG1 (non-A and A allotypes) (SEQ ID NOs: 3 and 4, respectively), humIgG2 (SEQ ID NO:5), humIgG3 (SEQ ID NO:6) and humIgG4 (SEQ ID NO:7), are shown. The human IgG1 sequence is the non-A allotype, and differences between this sequence and the A allotype (at positions 356 and 358; EU numbering system) are shown below the human IgG1 sequence. Native sequence murine IgG Fc region sequences, murIgG1 (SEQ

ID NO:8), murIgG2A (SEQ ID NO:9), murIgG2B (SEQ ID NO:10) and murIgG3 (SEQ ID NO:11), are also shown. Figure 22B shows percent identity among the Fc region sequences of Figure 22A.

Figure 23 depicts alignments of native sequence human IgG Fc region sequences, humIgG1 (non-A and A allotypes; SEQ ID NOs:3 and 4, respectively), humIgG2 (SEQ ID NO:5), humIgG3 (SEQ ID NO:6) and humIgG4 (SEQ ID NO:7) with differences between the sequences marked with asterisks.

Figure 24 shows area under curve (AUC) for selected variants compared to anti-HER2 IgG1 (HERCEPTIN[®]) in a 4 hour ADCC assay. The effector cells were PBMCs (N=5). Variant G36 (S317A; 298 in Eu numbering) with improved binding to FcγRIIIA showed improved ADCC activity; variant G31 (R309A; 292 in Eu numbering) which did not display altered FcγRIIIA binding, also had unaltered ADCC activity; and G14 (D265A; 278 in Eu numbering) which had reduced FcγRIIIA binding, also had reduced ADCC activity.

Figures 25A and B depict binding of anti-IgE E27 IgG1 to human FcγR. Figure 25A shows binding of E27 monomers (solid circles), dimeric (solid squares), and hexameric (open squares) complexes to FcγRIIIA-R131. Figure 25B shows binding of E27 monomers (solid circles), dimeric (solid squares), and hexameric (open squares) complexes to FcγRIIIA-F158. Dimers were formed by mixing E27 IgG1 and a F(ab')₂ fragment of goat anti-human k light chain at 1:0.5 molar ratio at 25°C for 1 hr (Huizinga *et al. J. Immunol.* 142: 2359-2364 (1989)). Hexameric complexes (*i.e.* trimeric in E27 IgG1) were formed by mixing E27 IgG1 with human IgE in a 1:1 molar ratio at 25°C for 1 hr (Liu *et al. Biochem.* 34:10474-10482 (1995)).

Figures 26 A and B show binding sites of human IgG1 for FcγR. Figure 26A depicts IgG1 residues comprising the binding site for FcγRI and FcγRII. The two Fc heavy chains are in *light* and *medium gray*; carbohydrate is in *dark gray*. Residues that affected binding to all FcγR are in *red*; the FcγRI binding site is comprised only of *red* residues. Residues that showed improved binding to FcγRII and FcγRIIIA are in *magenta*. Residues that showed reduced binding only to FcγRII are in *yellow*. Residues that showed ≥ 50% improved binding only to FcγRII are in *green*. Fig. 26B depicts IgG1 residues comprising the binding site for FcγRI and FcγRIIIA. The two Fc heavy chains are in *light* and *medium gray*; carbohydrate is in *dark gray*. Residues that affected binding to all FcγR are in *red*; the FcγRI binding site is comprised only of *red* residues. Residues that showed improved binding to FcγRII and FcγRIIIA are in *magenta*. Residues that showed reduced binding only to FcγRIIIA are in *yellow*. Residues that showed ≥ 25% improved binding only to FcγRIIIA are in *green*. Glu430, involved in a salt-bridge

with Lys338, is shown in *blue*.

Figures 27A-C show ADCC of anti-p¹⁸⁵HER2 IgG1 variants for FcγRIIIA-F158 and FcγRIIIA-V158 allotypes. ADCC was performed using p¹⁸⁵HER2-expressing SK-BR-3 cells as target and Natural Killer cells (NKs) isolated from three FcγRIIIA-Val158/Val158 donors or three FcγRIIIA-Phe158/Phe158 donors were used as effector cells. Cytotoxicity was detected by LDH release. Antibody-independent cell cytotoxicity (AICC) was measured using target and effector cells together (*i.e.* no antibody). Maximal release (MR) was measured by adding 1% Triton-100™ to target cells. Percent cytotoxicity was calculated as $(\text{LDH release}_{\text{sample}} / \text{MR}_{\text{target}}) \times 100$. The Effector:Target ratio was plotted versus % cytotoxicity. Figure 27A shows representative ADCC assay for IgG1 variants using NK cells from an FcγRIIIA-Val158/Val158 donor. Native anti-p¹⁸⁵HER2 IgG1 (solid circles), Ser298Ala (solid squares), Lys334Ala (solid triangles), Ser298Ala/Lys334Ala (open circles), Ser298Ala/Glu333Ala/Lys334Ala (open squares), AICC (open triangles). Figure 27B shows representative ADCC assay for IgG1 variants using NK cells from an FcγRIIIA-Phe158/Phe158 donor. Native anti-p¹⁸⁵HER2 IgG1 (solid circles), Ser298Ala (solid squares), Lys334Ala (solid triangles), Ser298Ala/Lys334Ala (open circles), Ser298Ala/Glu333Ala/Lys334Ala (open squares), AICC (open triangles). Figure 27C shows a bar plot of mean % increase in ADCC of variants compared to native anti-p¹⁸⁵HER2 IgG1. Percent increase was calculated as $(\% \text{ cytotoxicity}_{\text{variant}} - \% \text{ cytotoxicity}_{\text{native IgG1}}) / \% \text{ cytotoxicity}_{\text{native IgG1}}$. For each variant, the mean and standard deviation are for 13 data points using three different donors. For all variants the FcγRIIIA-Phe158/Phe158 donors showed a significant increase in ADCC over the increase seen for FcγRIIIA-Val158/Val158 donors ($P < 0.0001$ for all variants using paired t-test).

Detailed Description of the Preferred Embodiments

I. Definitions

Throughout the present specification and claims, the numbering of the residues in an immunoglobulin heavy chain is that of the EU index as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991), expressly incorporated herein by reference. The "EU index as in Kabat" refers to the residue numbering of the human IgG1 EU antibody.

A "parent polypeptide" is a polypeptide comprising an amino acid sequence which lacks one or more of the Fc region modifications disclosed herein and which differs in effector function compared to a polypeptide variant as herein disclosed. The parent polypeptide may comprise a native sequence Fc region or an Fc region with pre-existing amino acid sequence

modifications (such as additions, deletions and/or substitutions).

The term "Fc region" is used to define a C-terminal region of an immunoglobulin heavy chain, *e.g.*, as shown in Figure 1. The "Fc region" may be a native sequence Fc region or a variant Fc region. Although the boundaries of the Fc region of an immunoglobulin heavy chain might vary, the human IgG heavy chain Fc region is usually defined to stretch from an amino acid residue at position Cys226, or from Pro230, to the carboxyl-terminus thereof. The Fc region of an immunoglobulin generally comprises two constant domains, CH2 and CH3, as shown, for example, in Fig. 1.

The "CH2 domain" of a human IgG Fc region (also referred to as "C γ 2" domain) usually extends from about amino acid 231 to about amino acid 340. The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It has been speculated that the carbohydrate may provide a substitute for the domain-domain pairing and help stabilize the CH2 domain. Burton, *Molec. Immunol.*22:161-206 (1985).

The "CH3 domain" comprises the stretch of residues C-terminal to a CH2 domain in an Fc region (*i.e.* from about amino acid residue 341 to about amino acid residue 447 of an IgG)

A "functional Fc region" possesses an "effector function" of a native sequence Fc region. Exemplary "effector functions" include C1q binding; complement dependent cytotoxicity; Fc receptor binding; antibody-dependent cell-mediated cytotoxicity (ADCC); phagocytosis; down regulation of cell surface receptors (*e.g.* B cell receptor; BCR), etc. Such effector functions generally require the Fc region to be combined with a binding domain (*e.g.* an antibody variable domain) and can be assessed using various assays as herein disclosed, for example.

A "native sequence Fc region" comprises an amino acid sequence identical to the amino acid sequence of an Fc region found in nature. Native sequence human Fc regions are shown in Fig. 23 and include a native sequence human IgG1 Fc region (non-A and A allotypes); native sequence human IgG2 Fc region; native sequence human IgG3 Fc region; and native sequence human IgG4 Fc region as well as naturally occurring variants thereof. Native sequence murine Fc regions are shown in Fig. 22A.

A "variant Fc region" comprises an amino acid sequence which differs from that of a native sequence Fc region by virtue of at least one "amino acid modification" as herein defined.

Preferably, the variant Fc region has at least one amino acid substitution compared to a native sequence Fc region or to the Fc region of a parent polypeptide, *e.g.* from about one to about ten amino acid substitutions, and preferably from about one to about five amino acid substitutions in a native sequence Fc region or in the Fc region of the parent polypeptide. The variant Fc region herein will preferably possess at least about 80% homology with a native sequence Fc region and/or with an Fc region of a parent polypeptide, and most preferably at least about 90%

homology therewith, more preferably at least about 95% homology therewith.

"Homology" is defined as the percentage of residues in the amino acid sequence variant that are identical after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology. Methods and computer programs for the alignment are well known in the art. One such computer program is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, DC 20559, on December 10, 1991.

The term "Fc region-containing polypeptide" refers to a polypeptide, such as an antibody or immunoadhesin (see definitions below), which comprises an Fc region.

The terms "Fc receptor" or "FcR" are used to describe a receptor that binds to the Fc region of an antibody. The preferred FcR is a native sequence human FcR. Moreover, a preferred FcR is one which binds an IgG antibody (a gamma receptor) and includes receptors of the Fc γ RI, Fc γ RII, and Fc γ RIII subclasses, including allelic variants and alternatively spliced forms of these receptors. Fc γ RII receptors include Fc γ RIIA (an "activating receptor") and Fc γ RIIB (an "inhibiting receptor"), which have similar amino acid sequences that differ primarily in the cytoplasmic domains thereof. Activating receptor Fc γ RIIA contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic domain. Inhibiting receptor Fc γ RIIB contains an immunoreceptor tyrosine-based inhibition motif (ITIM) in its cytoplasmic domain. (see review M. in Daëron, *Annu. Rev. Immunol.* 15:203-234 (1997)). FcRs are reviewed in Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991); Capel *et al.*, *Immunomethods* 4:25-34 (1994); and de Haas *et al.*, *J. Lab. Clin. Med.* 126:330-41 (1995). Other FcRs, including those to be identified in the future, are encompassed by the term "FcR" herein. The term also includes the neonatal receptor, FcRn, which is responsible for the transfer of maternal IgGs to the fetus (Guyer *et al.*, *J. Immunol.* 117:587 (1976) and Kim *et al.*, *J. Immunol.* 24:249 (1994)). The term includes allotypes, such as Fc γ RIIIA allotypes: Fc γ RIIIA-Phe158, Fc γ RIIIA-Val158, Fc γ RIIA-R131 and/or Fc γ RIIA-H131.

Antibody-dependent cell-mediated cytotoxicity" and "ADCC" refer to a cell-mediated reaction in which nonspecific cytotoxic cells that express FcRs (*e.g.* Natural Killer (NK) cells, neutrophils, and macrophages) recognize bound antibody on a target cell and subsequently cause lysis of the target cell. The primary cells for mediating ADCC, NK cells, express Fc γ RIII only, whereas monocytes express Fc γ RI, Fc γ RII and Fc γ RIII. FcR expression on hematopoietic cells is summarized in Table 3 on page 464 of Ravetch and Kinet, *Annu. Rev. Immunol* 9:457-92 (1991).

"Human effector cells" are leukocytes which express one or more FcRs and perform effector functions. Preferably, the cells express at least Fc γ RIII and perform ADCC effector

function. Examples of human leukocytes which mediate ADCC include peripheral blood mononuclear cells (PBMC), natural killer (NK) cells, monocytes, cytotoxic T cells and neutrophils; with PBMCs and NK cells being preferred. The effector cells may be isolated from a native source thereof, *e.g.* from blood or PBMCs as described herein.

5 A polypeptide variant with "altered" FcR binding affinity or ADCC activity is one which has either enhanced or diminished FcR binding activity and/or ADCC activity compared to a parent polypeptide or to a polypeptide comprising a native sequence Fc region. The polypeptide variant which "displays increased binding" to an FcR binds at least one FcR with better affinity than the parent polypeptide. The improvement in binding compared to a parent polypeptide may be
10 about 25% or better improvement, *e.g.* up to about 200% or about 1000% improvement in binding. The polypeptide variant which "displays decreased binding" to an FcR, binds at least one FcR with worse affinity than a parent polypeptide. The decrease in binding compared to a parent polypeptide may be about 40% or more decrease in binding, *e.g.* down to a variant which possess little or no appreciable binding to the FcR. Such variants which display decreased
15 binding to an FcR may possess little or no appreciable binding to an FcR, *e.g.*, 0-20% binding to the FcR compared to a native sequence IgG Fc region, *e.g.* as determined in the Examples herein.

The polypeptide variant which binds an FcR with "better affinity" than a parent polypeptide, is one which binds any one or more of the above identified FcRs with substantially
20 better binding affinity than the parent antibody, when the amounts of polypeptide variant and parent polypeptide in the binding assay are essentially the same. For example, the polypeptide variant with improved FcR binding affinity may display from about 1.15 fold to about 100 fold, *e.g.* from about 1.2 fold to about 50 fold improvement in FcR binding affinity compared to the parent polypeptide, where FcR binding affinity is determined, for example, as disclosed in the Examples
25 herein.

The polypeptide variant which "mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more effectively" than a parent antibody is one which *in vitro* or *in vivo* is substantially more effective at mediating ADCC, when the amounts of polypeptide variant and parent antibody used in the assay are essentially the same. Generally,
30 such variants will be identified using the *in vitro* ADCC assay as herein disclosed, but other assays or methods for determining ADCC activity, *e.g.* in an animal model etc, are contemplated. The preferred variant is from about 1.5 fold to about 100 fold, *e.g.* from about two fold to about fifty fold, more effective at mediating ADCC than the parent, *e.g.* in the *in vitro* assay disclosed herein.

35 An "amino acid modification" refers to a change in the amino acid sequence of a predetermined amino acid sequence. Exemplary modifications include an amino acid

substitution, insertion and/or deletion. The preferred amino acid modification herein is a substitution.

An "amino acid modification at" a specified position, e.g. of the Fc region, refers to the substitution or deletion of the specified residue, or the insertion of at least one amino acid residue adjacent the specified residue. By insertion "adjacent" a specified residue is meant insertion within one to two residues thereof. The insertion may be N-terminal or C-terminal to the specified residue.

An "amino acid substitution" refers to the replacement of at least one existing amino acid residue in a predetermined amino acid sequence with another different "replacement" amino acid residue. The replacement residue or residues may be "naturally occurring amino acid residues" (i.e. encoded by the genetic code) and selected from the group consisting of: alanine (Ala); arginine (Arg); asparagine (Asn); aspartic acid (Asp); cysteine (Cys); glutamine (Gln); glutamic acid (Glu); glycine (Gly); histidine (His); isoleucine (Ile); leucine (Leu); lysine (Lys); methionine (Met); phenylalanine (Phe); proline (Pro); serine (Ser); threonine (Thr); tryptophan (Trp); tyrosine (Tyr); and valine (Val). Preferably, the replacement residue is not cysteine. Substitution with one or more non-naturally occurring amino acid residues is also encompassed by the definition of an amino acid substitution herein. A "non-naturally occurring amino acid residue" refers to a residue, other than those naturally occurring amino acid residues listed above, which is able to covalently bind adjacent amino acid residues(s) in a polypeptide chain. Examples of non-naturally occurring amino acid residues include norleucine, ornithine, norvaline, homoserine and other amino acid residue analogues such as those described in Ellman *et al. Meth. Enzym.* 202:301-336 (1991). To generate such non-naturally occurring amino acid residues, the procedures of Noren *et al. Science* 244:182 (1989) and Ellman *et al., supra*, can be used. Briefly, these procedures involve chemically activating a suppressor tRNA with a non-naturally occurring amino acid residue followed by *in vitro* transcription and translation of the RNA.

An "amino acid insertion" refers to the incorporation of at least one amino acid into a predetermined amino acid sequence. While the insertion will usually consist of the insertion of one or two amino acid residues, the present application contemplates larger "peptide insertions", e.g. insertion of about three to about five or even up to about ten amino acid residues. The inserted residue(s) may be naturally occurring or non-naturally occurring as disclosed above.

An "amino acid deletion" refers to the removal of at least one amino acid residue from a predetermined amino acid sequence.

"Hinge region" is generally defined as stretching from Glu216 to Pro230 of human IgG1 (Burton, *Molec. Immunol.* 22:161-206 (1985)). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain S-S bonds in the same positions.

The "lower hinge region" of an Fc region is normally defined as the stretch of residues immediately C-terminal to the hinge region, *i.e.* residues 233 to 239 of the Fc region. Prior to the present invention, Fc γ R binding was generally attributed to amino acid residues in the lower hinge region of an IgG Fc region.

5 "C1q" is a polypeptide that includes a binding site for the Fc region of an immunoglobulin. C1q together with two serine proteases, C1r and C1s, forms the complex C1, the first component of the complement dependent cytotoxicity (CDC) pathway. Human C1q can be purchased commercially from, *e.g.* Quidel, San Diego, CA.

10 The term "binding domain" refers to the region of a polypeptide that binds to another molecule. In the case of an FcR, the binding domain can comprise a portion of a polypeptide chain thereof (*e.g.* the α chain thereof) which is responsible for binding an Fc region. One useful binding domain is the extracellular domain of an FcR α chain.

15 The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (*e.g.*, bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

20 "Antibody fragments", as defined for the purpose of the present invention, comprise a portion of an intact antibody, generally including the antigen binding or variable region of the intact antibody or the Fc region of an antibody which retains FcR binding capability. Examples of antibody fragments include linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. The antibody fragments preferably retain at least part of the hinge and optionally the CH1 region of an IgG heavy chain. More preferably, the antibody fragments retain the entire constant region of an IgG heavy chain, and include an IgG light chain.

25 The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in
35 accordance with the present invention may be made by the hybridoma method first described

by Kohler *et al.*, *Nature* 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in Clackson *et al.*, *Nature* 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.* 222:581-597 (1991), for example.

5 The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another
10 species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Patent No. 4,816,567; and Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

 "Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. For the most part,
15 humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues.

20 Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions
25 are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones *et al.*, *Nature* 321:522-525 (1986); Riechmann *et al.*, *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992).

 The term "hypervariable region" when used herein refers to the amino acid residues of
30 an antibody which are responsible for antigen-binding. The hypervariable region comprises amino acid residues from a "complementarity determining region" or "CDR" (*i.e.* residues 24-34 (L1), 50-56 (L2) and 89-97 (L3) in the light chain variable domain and 31-35 (H1), 50-65 (H2) and 95-102 (H3) in the heavy chain variable domain; Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda,
35 MD. (1991)) and/or those residues from a "hypervariable loop" (*i.e.* residues 26-32 (L1), 50-52 (L2) and 91-96 (L3) in the light chain variable domain and 26-32 (H1), 53-55 (H2) and 96-101

(H3) in the heavy chain variable domain; Chothia and Lesk *J. Mol. Biol.* 196:901-917 (1987)). "Framework" or "FR" residues are those variable domain residues other than the hypervariable region residues as herein defined.

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the "binding domain" of a heterologous "adhesin" protein (*e.g.* a receptor, ligand or enzyme) with an immunoglobulin constant domain. Structurally, the immunoadhesins comprise a fusion of the adhesin amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (*i.e.* is "heterologous") and an immunoglobulin constant domain sequence.

The term "ligand binding domain" as used herein refers to any native cell-surface receptor or any region or derivative thereof retaining at least a qualitative ligand binding ability of a corresponding native receptor. In a specific embodiment, the receptor is from a cell-surface polypeptide having an extracellular domain that is homologous to a member of the immunoglobulin supergenefamily. Other receptors, which are not members of the immunoglobulin supergenefamily but are nonetheless specifically covered by this definition, are receptors for cytokines, and in particular receptors with tyrosine kinase activity (receptor tyrosine kinases), members of the hematopoietin and nerve growth factor receptor superfamilies, and cell adhesion molecules, *e.g.* (E-, L- and P-) selectins.

The term "receptor binding domain" is used to designate any native ligand for a receptor, including cell adhesion molecules, or any region or derivative of such native ligand retaining at least a qualitative receptor binding ability of a corresponding native ligand. This definition, among others, specifically includes binding sequences from ligands for the above-mentioned receptors.

An "antibody-immunoadhesin chimera" comprises a molecule that combines at least one binding domain of an antibody (as herein defined) with at least one immunoadhesin (as defined in this application). Exemplary antibody-immunoadhesin chimeras are the bispecific CD4-IgG chimeras described in Berg *et al.*, *PNAS (USA)* 88:4723-4727 (1991) and Chamow *et al.*, *J. Immunol.* 153:4268 (1994).

An "isolated" polypeptide is one that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to greater than 95% by weight of polypeptide as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to

homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes the polypeptide *in situ* within recombinant cells since at least one component of the polypeptide's natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification
5 step.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented.

10 A "disorder" is any condition that would benefit from treatment with the polypeptide variant. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. In one embodiment, the disorder is cancer.

The terms "cancer" and "cancerous" refer to or describe the physiological condition in mammals that is typically characterized by unregulated cell growth. Examples of cancer include
15 but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particular examples of such cancers include squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, adenocarcinoma of the lung, squamous carcinoma of the lung, cancer of the peritoneum, hepatocellular cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, liver cancer, bladder cancer, hepatoma, breast cancer, colon
20 cancer, colorectal cancer, endometrial or uterine carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer.

A "HER2-expressing cancer" is one comprising cells which have HER2 receptor protein (Semba *et al.*, *PNAS (USA)* 82:6497-6501 (1985) and Yamamoto *et al.* *Nature* 319:230-234
25 (1986) (Genebank accession number X03363)) present at their cell surface, such that an anti-HER2 antibody is able to bind to the cancer.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly to the polypeptide. The label may be itself be detectable (e.g., radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may
30 catalyze chemical alteration of a substrate compound or composition which is detectable.

An "isolated" nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the polypeptide nucleic acid. An isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid
35 molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated nucleic acid molecule includes a nucleic acid molecule contained in cells

that ordinarily express the polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The expression "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous.

Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny. Thus, the words "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Mutant progeny that have the same function or biological activity as screened for in the originally transformed cell are included. Where distinct designations are intended, it will be clear from the context.

The term "molecular complex" when used herein refers to the relatively stable structure which forms when two or more heterologous molecules (*e.g.* polypeptides) bind (preferably noncovalently) to one another. The preferred molecular complex herein is an immune complex.

"Immune complex" refers to the relatively stable structure which forms when at least one target molecule and at least one heterologous Fc region-containing polypeptide bind to one another forming a larger molecular weight complex. Examples of immune complexes are antigen-antibody aggregates and target molecule-immunoadhesin aggregates. The term "immune complex" as used herein, unless indicated otherwise, refers to an *ex vivo* complex (*i.e.* other than the form or setting in which it may be found in nature). However, the immune complex may be administered to a mammal, *e.g.* to evaluate clearance of the immune complex in the mammal.

The term "target molecule" refers to a molecule, usually a polypeptide, which is capable

of being bound by a heterologous molecule and has one or more binding sites for the heterologous molecule. The term "binding site" refers to a region of a molecule to which another molecule can bind. The "first target molecule" herein comprises at least two distinct binding sites (for example, two to five separate binding sites) for an analyte (*e.g.* an Fc region-containing polypeptide) such that at least two analyte molecules can bind to the first target molecule. In the preferred embodiment of the invention, the two or more binding sites are identical (*e.g.* having the same amino acid sequence, where the target molecule is a polypeptide). In Example 1 below, the first target molecule was IgE and had two separate binding sites in the Fc region thereof to which the Fc region-containing polypeptide (an anti-IgE antibody, E27) could bind.

Other first target molecules include dimers of substantially identical monomers (*e.g.* neurotrophins, IL8 and VEGF) or are polypeptides comprising two or more substantially identical polypeptide chains (*e.g.* antibodies or immunoadhesins). The "second target molecule" comprises at least two distinct binding sites (for example, two to five separate binding sites) for the first target molecule such that at least two first target molecules can bind to the second target molecule. Preferably, the two or more binding sites are identical (*e.g.* having the same amino acid sequence, where the target molecule is a polypeptide). In Example 2, the second target molecule was VEGF, which has a pair of distinct binding sites to which the variable domain of the IgE antibody could bind. Other second target molecules are contemplated, *e.g.* other dimers of substantially identical monomers (*e.g.* neurotrophins or IL8) or polypeptides comprising two or more substantially identical domains (*e.g.* antibodies or immunoadhesins).

An "analyte" is a substance that is to be analyzed. The preferred analyte is an Fc region-containing polypeptide that is to be analyzed for its ability to bind to an Fc receptor.

A "receptor" is a polypeptide capable of binding at least one ligand. The preferred receptor is a cell-surface receptor having an extracellular ligand-binding domain and, optionally, other domains (*e.g.* transmembrane domain, intracellular domain and/or membrane anchor). The receptor to be evaluated in the assay described herein may be an intact receptor or a fragment or derivative thereof (*e.g.* a fusion protein comprising the binding domain of the receptor fused to one or more heterologous polypeptides). Moreover, the receptor to be evaluated for its binding properties may be present in a cell or isolated and optionally coated on an assay plate or some other solid phase.

The phrase "low affinity receptor" denotes a receptor that has a weak binding affinity for a ligand of interest, *e.g.* having a binding constant of about 50nM or worse affinity. Exemplary low affinity receptors include FcγRII and FcγRIII.

II. Modes for Carrying Out the Invention

The invention herein relates to a method for making a polypeptide variant. The "parent", "starting" or "nonvariant" polypeptide is prepared using techniques available in the art for

generating polypeptides comprising an Fc region. In the preferred embodiment of the invention, the parent polypeptide is an antibody and exemplary methods for generating antibodies are described in more detail in the following sections. The parent polypeptide may, however, be any other polypeptide comprising an Fc region, *e.g.* an immunoadhesin. Methods for making immunoadhesins are elaborated in more detail hereinbelow.

In an alternative embodiment, a variant Fc region may be generated according to the methods herein disclosed and this "variant Fc region" can be fused to a heterologous polypeptide of choice, such as an antibody variable domain or binding domain of a receptor or ligand.

The parent polypeptide comprises an Fc region. Generally the Fc region of the parent polypeptide will comprise a native sequence Fc region, and preferably a human native sequence Fc region. However, the Fc region of the parent polypeptide may have one or more pre-existing amino acid sequence alterations or modifications from a native sequence Fc region. For example, the C1q binding activity of the Fc region may have been previously altered (other types of Fc region modifications are described in more detail below). In a further embodiment the parent polypeptide Fc region is "conceptual" and, while it does not physically exist, the antibody engineer may decide upon a desired variant Fc region amino acid sequence and generate a polypeptide comprising that sequence or a DNA encoding the desired variant Fc region amino acid sequence.

In the preferred embodiment of the invention, however, a nucleic acid encoding an Fc region of a parent polypeptide is available and this nucleic acid sequence is altered to generate a variant nucleic acid sequence encoding the Fc region variant.

DNA encoding an amino acid sequence variant of the starting polypeptide is prepared by a variety of methods known in the art. These methods include, but are not limited to, preparation by site-directed (or oligonucleotide-mediated) mutagenesis, PCR mutagenesis, and cassette mutagenesis of an earlier prepared DNA encoding the polypeptide

Site-directed mutagenesis is a preferred method for preparing substitution variants. This technique is well known in the art (see, *e.g.*, Carter *et al.* *Nucleic Acids Res.* 13:4431-4443 (1985) and Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488 (1987)). Briefly, in carrying out site-directed mutagenesis of DNA, the starting DNA is altered by first hybridizing an oligonucleotide encoding the desired mutation to a single strand of such starting DNA. After hybridization, a DNA polymerase is used to synthesize an entire second strand, using the hybridized oligonucleotide as a primer, and using the single strand of the starting DNA as a template. Thus, the oligonucleotide encoding the desired mutation is incorporated in the resulting double-stranded DNA.

PCR mutagenesis is also suitable for making amino acid sequence variants of the

starting polypeptide. See Higuchi, in *PCR Protocols*, pp.177-183 (Academic Press, 1990); and Vallette *et al.*, *Nuc. Acids Res.* 17:723-733 (1989). Briefly, when small amounts of template DNA are used as starting material in a PCR, primers that differ slightly in sequence from the corresponding region in a template DNA can be used to generate relatively large quantities of a specific DNA fragment that differs from the template sequence only at the positions where the primers differ from the template.

Another method for preparing variants, cassette mutagenesis, is based on the technique described by Wells *et al.*, *Gene* 34:315-323 (1985). The starting material is the plasmid (or other vector) comprising the starting polypeptide DNA to be mutated. The codon(s) in the starting DNA to be mutated are identified. There must be a unique restriction endonuclease site on each side of the identified mutation site(s). If no such restriction sites exist, they may be generated using the above-described oligonucleotide-mediated mutagenesis method to introduce them at appropriate locations in the starting polypeptide DNA. The plasmid DNA is cut at these sites to linearize it. A double-stranded oligonucleotide encoding the sequence of the DNA between the restriction sites but containing the desired mutation(s) is synthesized using standard procedures, wherein the two strands of the oligonucleotide are synthesized separately and then hybridized together using standard techniques. This double-stranded oligonucleotide is referred to as the cassette. This cassette is designed to have 5' and 3' ends that are compatible with the ends of the linearized plasmid, such that it can be directly ligated to the plasmid. This plasmid now contains the mutated DNA sequence.

Alternatively, or additionally, the desired amino acid sequence encoding a polypeptide variant can be determined, and a nucleic acid sequence encoding such amino acid sequence variant can be generated synthetically.

The amino acid sequence of the parent polypeptide is modified in order to generate a variant Fc region with altered Fc receptor binding affinity or activity *in vitro* and/or *in vivo* and/or altered antibody-dependent cell-mediated cytotoxicity (ADCC) activity *in vitro* and/or *in vivo*.

Generally, the modification entails one or more amino acid substitutions. In one embodiment, the replacement residue does not correspond to a residue present in the same position in any of the native sequence Fc regions in Figure 22A. For example, according to this embodiment of the invention, Pro331 of a human IgG3 or IgG1 Fc region is replaced with a residue other than Ser (the corresponding aligned residue found in native sequence human IgG4). In one embodiment, the residue in the parent polypeptide which is substituted with a replacement residue is not an alanine and/or is not residue Ala339 of an Fc region. In the case of an amino acid substitution, preferably the residue in the parent polypeptide is replaced with an alanine residue. However, the present invention contemplates replacement of the residue of the parent polypeptide with any other amino acid residue. The substitution may, for example,

be a "conservative substitution". Such conservative substitutions are shown in Table 1 under the heading of "preferred substitution". More substantial changes may be achieved by making one or more "exemplary substitutions" which are not the preferred substitution in Table 1.

5

TABLE 1

Original Residue	Exemplary Substitutions	Preferred Substitution
Ala (A)	val; leu; ile	val
Arg (R)	lys; gln; asn	lys
Asn (N)	gln; his; lys; arg	gln
Asp (D)	glu	glu
Cys (C)	ser	ser
Gln (Q)	asn	asn
Glu (E)	asp	asp
Gly (G)	pro; ala	ala
His (H)	asn; gln; lys; arg	arg
Ile (I)	leu; val; met; ala; phe; norleucine	leu
Leu (L)	norleucine; ile; val; met; ala; phe	ile
Lys (K)	arg; gln; asn	arg
Met (M)	leu; phe; ile	leu
Phe (F)	leu; val; ile; ala; tyr	leu
Pro (P)	ala	ala
Ser (S)	thr	thr
Thr (T)	ser	ser
Trp (W)	tyr; phe	tyr
Tyr (Y)	trp; phe; thr; ser	phe
Val (V)	ile; leu; met; phe; ala; norleucine	leu

Substantial modifications in the biological properties of the Fc region may be accomplished by selecting substitutions that differ significantly in their effect on maintaining (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Naturally occurring residues are divided into groups based on

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common side-chain properties:

- (1) hydrophobic: norleucine, met, ala, val, leu, ile;
- (2) neutral hydrophilic: cys, ser, thr;
- (3) acidic: asp, glu;
- 5 (4) basic: asn, gln, his, lys, arg;
- (5) residues that influence chain orientation: gly, pro; and
- (6) aromatic: trp, tyr, phe.

Non-conservative substitutions will entail exchanging a member of one of these classes for a member of another class. Conservative and non-conservative amino acid substitutions are
10 exemplified in Table 7 hereinbelow.

As is demonstrated in Example 4 herein, one can engineer an Fc region variant with altered binding affinity for one or more FcRs. As was shown in that Example, different classes of Fc region variants can be made *e.g.*, as summarized in the following table. Where the variant Fc region has more than one amino acid substitution, generally, but not necessarily, amino acid
15 substitutions in the same class are combined to achieve the desired result.

TABLE 2
CLASSES OF Fc REGION VARIANTS

Class	FcR binding property	Position of Fc region substitution(s)
1	reduced binding to all Fc γ R	233-236#, 238, 265#, 297#*, 327Gln, 329
2	reduced binding to both Fc γ RII and Fc γ RIII	270, 295, 327Ser
3	improved binding to both Fc γ RII and Fc γ RIII	256, 290
4	improved binding to Fc γ RII and no effect on Fc γ RIII binding	255, 258, 267, 272, 276, 280, 285, 286, 307, 309, 315, 326, 331, 337, 378, 430
5	improved binding to Fc γ RII and reduced binding to Fc γ RIII	268, 301, 322
6	reduced binding to Fc γ RII and no effect on Fc γ RIII binding	292, 414
7	reduced binding to Fc γ RII and improved binding to Fc γ RIII	298
8	no effect on Fc γ RII binding and reduced binding to Fc γ RIII	239, 269, 293, 296, 303, 327Gly, 338, 376
9	no effect on Fc γ RII binding and	333, 334, 339#

	improved binding to Fc γ RIII	
10	effect only FcRn	253, 254, 288, 305, 311, 312, 317, 360, 362, 380, 382, 415, 424, 433, 434, 435, 436

* deglycosylated version

Preferably combined with other Fc modification(s), (e.g. as disclosed herein)

Aside from amino acid substitutions, the present invention contemplates other modifications of the parent Fc region amino acid sequence in order to generate an Fc region variant with altered effector function.

One may, for example, delete one or more amino acid residues of the Fc region in order to reduce binding to an FcR. Generally, one will delete one or more of the Fc region residues identified herein as effecting FcR binding (see Example 4 below) in order to generate such an Fc region variant. Generally, no more than one to about ten Fc region residues will be deleted according to this embodiment of the invention. The Fc region herein comprising one or more amino acid deletions will preferably retain at least about 80%, and preferably at least about 90%, and most preferably at least about 95%, of the parent Fc region or of a native sequence human Fc region.

One may also make amino acid insertion Fc region variants, which variants have altered effector function. For example, one may introduce at least one amino acid residue (e.g. one to two amino acid residues and generally no more than ten residues) adjacent to one or more of the Fc region positions identified herein as impacting FcR binding. By "adjacent" is meant within one to two amino acid residues of a Fc region residue identified herein. Such Fc region variants may display enhanced or diminished FcR binding and/or ADCC activity. In order to generate such insertion variants, one may evaluate a co-crystal structure of a polypeptide comprising a binding region of an FcR (e.g. the extracellular domain of the FcR of interest) and the Fc region into which the amino acid residue(s) are to be inserted (see, for example, Deisenhofer, *Biochemistry* 20(9):2361-2370 (1981); and Burmeister *et al.*, *Nature* 342:379-383, (1994)) in order to rationally design an Fc region variant with, e.g., improved FcR binding ability. Such insertion(s) will generally be made in an Fc region loop, but not in the secondary structure (i.e. in a β -strand) of the Fc region.

By introducing the appropriate amino acid sequence modifications in a parent Fc region, one can generate a variant Fc region which (a) mediates antibody-dependent cell-mediated cytotoxicity (ADCC) in the presence of human effector cells more effectively and/or (b) binds an Fc gamma receptor (Fc γ R) with better affinity than the parent polypeptide. Such Fc region variants will generally comprise at least one amino acid modification in the Fc region. Combining amino acid modifications is thought to be particularly desirable. For example, the variant Fc

region may include two, three, four, five, etc substitutions therein, *e.g.* of the specific Fc region positions identified herein.

Preferably, the parent polypeptide Fc region is a human Fc region, *e.g.* a native sequence human Fc region human IgG1 (A and non-A allotypes), IgG2, IgG3 or IgG4 Fc region.

5 Such sequences are shown in Fig. 23.

To generate an Fc region with improved ADCC activity, the parent polypeptide preferably has pre-existing ADCC activity, *e.g.*, it comprises a human IgG1 or human IgG3 Fc region. In one embodiment, the variant with improved ADCC mediates ADCC substantially more effectively than an antibody with a native sequence IgG1 or IgG3 Fc region and the antigen-binding region
 10 of the variant. Preferably, the variant comprises, or consists essentially of, substitutions of two or three or all of the residues at positions 298, 333, 334 and 339 of the Fc region. Most preferably, residues at positions 298, 333, 334 and 339 are substituted, (*e.g.* with alanine residues). At position 298, the preferred replacement residue is an ala or gly, and most preferably ala. The preferred replacement residue at position 333 is ala or asp, and most
 15 preferably ala. The residue at position 334 is preferably replaced by one of the following amino acids: ala, gln, glu, met, tyr, his, val, leu, asn, ser, trp, with ala or glu being the most preferred replacement residues at this position. The preferred replacement residue at position 339 is a thr. Moreover, in order to generate the Fc region variant with improved ADCC activity, one will generally engineer an Fc region variant with improved binding affinity for FcγRIII, which is
 20 thought to be an important FcR for mediating ADCC. For example, one may introduce an amino acid modification (*e.g.* a substitution) into the parent Fc region at any one or more of amino acid positions 256, 290, 298, 312, 326, 330, 333, 334, 360, 378 or 430 to generate such a variant. The variant with improved binding affinity for FcγRIII may further have reduced binding affinity for FcγRII, especially reduced affinity for the inhibiting FcγRIIB receptor.

25 The amino acid modification(s) are preferably introduced into the CH2 domain of a Fc region, since the experiments herein indicate that the CH2 domain is important for FcR binding activity. Moreover, unlike the teachings of the above-cited art, the instant application contemplates the introduction of a modification into a part of the Fc region other than in the lower hinge region thereof.

30 Useful amino acid positions for modification in order to generate a variant IgG Fc region with altered Fc gamma receptor (FcγR) binding affinity or activity include any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 255, 256, 258, 265, 267, 268, 269, 270, 272, 276, 278, 280, 283, 285, 286, 289, 290, 292, 293, 294, 295, 296, 298, 301, 303, 305, 307, 309, 312, 315, 320, 322, 324, 326, 327, 329, 330, 331, 333, 334, 335, 337, 338, 340, 360, 373, 376,
 35 378, 382, 388, 389, 398, 414, 416, 419, 430, 434, 435, 437, 438 or 439 of the Fc region.

Preferably, the parent Fc region used as the template to generate such variants comprises a human IgG Fc region. Where residue 331 is substituted, the parent Fc region is preferably not human native sequence IgG3, or the variant Fc region comprising a substitution at position 331 preferably displays increased FcR binding, e.g. to FcγRII.

5 To generate an Fc region variant with reduced binding to the FcγR one may introduce an amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 292, 293, 294, 295, 296, 298, 301, 303, 322, 324, 327, 329, 333, 335, 338, 340, 373, 376, 382, 388, 389, 414, 416, 419, 434, 435, 437, 438 or 439 of the Fc region.

10 Variants which display reduced binding to FcγRI, include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 327 or 329.

15 Variants which display reduced binding to FcγRII include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 265, 269, 270, 292, 294, 295, 298, 303, 324, 327, 329, 333, 335, 338, 373, 376, 414, 416, 419, 435, 438 or 439.

Fc region variants which display reduced binding to FcγRIII include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 239, 248, 249, 252, 254, 265, 268, 269, 270, 272, 278, 289, 293, 294, 295, 296, 301, 303, 322, 327, 329, 338, 340, 373, 376, 382, 388, 389, 416, 434, 435 or 437 .

20 Variants with improved binding to one or more FcγRs may also be made. Such Fc region variants may comprise an amino acid modification at any one or more of amino acid positions 255, 256, 258, 267, 268, 272, 276, 280, 283, 285, 286, 290, 298, 301, 305, 307, 309, 312, 315, 320, 322, 326, 330, 331, 333, 334, 337, 340, 360, 378, 398 or 430 of the Fc region.

25 For example, the variant with improved FcγR binding activity may display increased binding to FcγRIII, and optionally may further display decreased binding to FcγRII; e.g. the variant may comprise an amino acid modification at position 298 and/or 333 of an Fc region.

30 Variants with increased binding to FcγRII include those comprising an amino acid modification at any one or more of amino acid positions 255, 256, 258, 267, 268, 272, 276, 280, 283, 285, 286, 290, 301, 305, 307, 309, 312, 315, 320, 322, 326, 330, 331, 337, 340, 378, 398 or 430 of an Fc region. Such variants may further display decreased binding to FcγRIII. For example, they may include an Fc region amino acid modification at any one or more of amino acid positions 268, 272, 298, 301, 322 or 340.

35 While it is preferred to alter binding to a FcγR, Fc region variants with altered binding affinity for the neonatal receptor (FcRn) are also contemplated herein. Fc region variants with improved affinity for FcRn are anticipated to have longer serum half-lives, and such molecules

will have useful applications in methods of treating mammals where long half-life of the administered polypeptide is desired, *e.g.*, to treat a chronic disease or disorder. Fc region variants with decreased FcRn binding affinity, on the contrary, are expected to have shorter half-lives, and such molecules may, for example, be administered to a mammal where a shortened circulation time may be advantageous, *e.g.* for *in vivo* diagnostic imaging or for polypeptides which have toxic side effects when left circulating in the blood stream for extended periods, etc. Fc region variants with decreased FcRn binding affinity are anticipated to be less likely to cross the placenta, and thus may be utilized in the treatment of diseases or disorders in pregnant women.

Fc region variants with altered binding affinity for FcRn include those comprising an Fc region amino acid modification at any one or more of amino acid positions 238, 252, 253, 254, 255, 256, 265, 272, 286, 288, 303, 305, 307, 309, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 386, 388, 400, 413, 415, 424, 433, 434, 435, 436, 439 or 447. Those which display reduced binding to FcRn will generally comprise an Fc region amino acid modification at any one or more of amino acid positions 252, 253, 254, 255, 288, 309, 386, 388, 400, 415, 433, 435, 436, 439 or 447; and those with increased binding to FcRn will usually comprise an Fc region amino acid modification at any one or more of amino acid positions 238, 256, 265, 272, 286, 303, 305, 307, 311, 312, 317, 340, 356, 360, 362, 376, 378, 380, 382, 413, 424 or 434.

The polypeptide variant(s) prepared as described above may be subjected to further modifications, oftentimes depending on the intended use of the polypeptide. Such modifications may involve further alteration of the amino acid sequence (substitution, insertion and/or deletion of amino acid residues), fusion to heterologous polypeptide(s) and/or covalent modifications. Such "further modifications" may be made prior to, simultaneously with, or following, the amino acid modification(s) disclosed above which result in an alteration of Fc receptor binding and/or ADCC activity. In one embodiment, one may combine the Fc region modification herein with Fc region substitutions disclosed in the references cited in the "Related Art" section of this application.

Alternatively or additionally, it may be useful to combine the above amino acid modifications with one or more further amino acid modifications that alter FcRn binding and/or half-life of the antibody and/or C1q binding and/or complement dependent cytotoxicity function of the Fc region.

The starting polypeptide of particular interest herein is usually one that binds to C1q and displays complement dependent cytotoxicity (CDC). The further amino acid substitutions described herein will generally serve to alter the ability of the starting polypeptide to bind to C1q and/or modify its complement dependent cytotoxicity function, *e.g.* to reduce and preferably abolish these effector functions. However, polypeptides comprising substitutions at one or more

of the described positions with improved C1q binding and/or complement dependent cytotoxicity (CDC) function are contemplated herein. For example, the starting polypeptide may be unable to bind C1q and/or mediate CDC and may be modified according to the teachings herein such that it acquires these further effector functions. Moreover, polypeptides with pre-existing C1q binding activity, optionally further having the ability to mediate CDC may be modified such that one or both of these activities are enhanced.

To generate an Fc region with altered C1q binding and/or complement dependent cytotoxicity (CDC) function, the amino acid positions to be modified are generally selected from heavy chain positions 270, 322, 326, 327, 329, 331, 333, and 334, where the numbering of the residues in an IgG heavy chain is that of the EU index as in Kabat *et al.*, *Sequences of Proteins of Immunological Interest*, 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD (1991). In one embodiment, only one of the eight above-identified positions is altered in order to generate the polypeptide variant region with altered C1q binding and/or complement dependent cytotoxicity (CDC) function. Preferably only residue 270, 329 or 322 is altered if this is the case. Alternatively, two or more of the above-identified positions are modified. If substitutions are to be combined, generally substitutions which enhance human C1q binding (e.g. at residue positions 326, 327, 333 and 334) or those which diminish human C1q binding (e.g., at residue positions 270, 322, 329 and 331) are combined. In the latter embodiment, all four positions (i.e., 270, 322, 329 and 331) may be substituted. Preferably, further substitutions at two, three or all of positions 326, 327, 333 or 334 are combined, optionally with other Fc region substitutions, to generate a polypeptide with improved human C1q binding and preferably improved CDC activity *in vitro* or *in vivo*.

Proline is conserved at position 329 in human IgG's. This residue is preferably replaced with alanine, however substitution with any other amino acid is contemplated, e.g., serine, threonine, asparagine, glycine or valine.

Proline is conserved at position 331 in human IgG1, IgG2 and IgG3, but not IgG4 (which has a serine residue at position 331). Residue 331 is preferably replaced by alanine or another amino acid, e.g. serine (for IgG regions other than IgG4), glycine or valine.

Lysine 322 is conserved in human IgGs, and this residue is preferably replaced by an alanine residue, but substitution with any other amino acid residue is contemplated, e.g. serine, threonine, glycine or valine.

D270 is conserved in human IgGs, and this residue may be replaced by another amino acid residue, e.g. alanine, serine, threonine, glycine, valine, or lysine.

K326 is also conserved in human IgGs. This residue may be substituted with another residue including, but not limited to, valine, glutamic acid, alanine, glycine, aspartic acid, methionine or tryptophan, with tryptophan being preferred.

Likewise, E333 is also conserved in human IgGs. E333 is preferably replaced by an amino acid residue with a smaller side chain volume, such as valine, glycine, alanine or serine, with serine being preferred.

K334 is conserved in human IgGs and may be substituted with another residue such as alanine or other residue.

In human IgG1 and IgG3, residue 327 is an alanine. In order to generate a variant with improved C1q binding, this alanine may be substituted with another residue such as glycine. In IgG2 and IgG4, residue 327 is a glycine and this may be replaced by alanine (or another residue) to diminish C1q binding.

As disclosed above, one can design an Fc region with altered effector function, *e.g.*, by modifying C1q binding and/or FcR binding and thereby changing CDC activity and/or ADCC activity. For example, one can generate a variant Fc region with improved C1q binding and improved FcγRIII binding; *e.g.* having both improved ADCC activity and improved CDC activity.

Alternatively, where one desires that effector function be reduced or ablated, one may engineer a variant Fc region with reduced CDC activity and/or reduced ADCC activity. In other embodiments, one may increase only one of these activities, and optionally also reduce the other activity, *e.g.* to generate an Fc region variant with improved ADCC activity, but reduced CDC activity *and vice versa*.

With respect to further amino acid sequence alterations, any cysteine residue not involved in maintaining the proper conformation of the polypeptide variant also may be substituted, generally with serine, to improve the oxidative stability of the molecule and prevent aberrant cross linking.

Another type of amino acid substitution serves to alter the glycosylation pattern of the polypeptide. This may be achieved by deleting one or more carbohydrate moieties found in the polypeptide, and/or adding one or more glycosylation sites that are not present in the polypeptide. Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-aceylgalactosamine, galactose, or xylose to a hydroxyamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used. Addition of glycosylation sites to the polypeptide is conveniently accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the

addition of, or substitution by, one or more serine or threonine residues to the sequence of the original polypeptide (for O-linked glycosylation sites). An exemplary glycosylation variant has an amino acid substitution of residue Asn 297 of the heavy chain.

Moreover, the class, subclass or allotype of the Fc region may be altered by one or more
5 further amino acid substitutions to generate an Fc region with an amino acid sequence more homologous to a different class, subclass or allotype as desired. For example, a murine Fc region may be altered to generate an amino acid sequence more homologous to a human Fc region; a human non-A allotype IgG1 Fc region may be modified to achieve a human A allotype IgG1 Fc region etc. In one embodiment, the amino modification(s) herein which alter FcR
10 binding and/or ADCC activity are made in the CH2 domain of the Fc region and the CH3 domain is deleted or replaced with another dimerization domain. Preferably, however, the CH3 domain is retained (aside from amino acid modifications therein which alter effector function as herein disclosed).

The polypeptide variant may be subjected to one or more assays to evaluate any change
15 in biological activity compared to the starting polypeptide.

Preferably the polypeptide variant essentially retains the ability to bind antigen compared to the nonvariant polypeptide, *i.e.* the binding capability is no worse than about 20 fold, *e.g.* no worse than about 5 fold of that of the nonvariant polypeptide. The binding capability of the polypeptide variant may be determined using techniques such as fluorescence activated cell
20 sorting (FACS) analysis or radioimmunoprecipitation (RIA), for example.

The ability of the polypeptide variant to bind an FcR may be evaluated. Where the FcR is a high affinity Fc receptor, such as FcγRI, FcRn or FcγRIIIA-V158, binding can be measured by titrating monomeric polypeptide variant and measuring bound polypeptide variant using an antibody which specifically binds to the polypeptide variant in a standard ELISA format (see
25 Example 2 below). Another FcR binding assay for low affinity FcRs is described in Examples 1 and 4.

To assess ADCC activity of the polypeptide variant, an *in vitro* ADCC assay, such as that described in Example 4 may be performed using varying effector:target ratios. Useful "effector cells" for such assays include peripheral blood mononuclear cells (PBMC) and Natural Killer
30 (NK) cells. Alternatively, or additionally, ADCC activity of the polypeptide variant may be assessed *in vivo*, *e.g.*, in a animal model such as that disclosed in Clynes *et al. PNAS (USA)* 95:652-656 (1998).

The ability of the variant to bind C1q and mediate complement dependent cytotoxicity (CDC) may be assessed.

35 To determine C1q binding, a C1q binding ELISA may be performed. Briefly, assay plates may be coated overnight at 4°C with polypeptide variant or starting polypeptide (control) in

coating buffer. The plates may then be washed and blocked. Following washing, an aliquot of human C1q may be added to each well and incubated for 2 hrs at room temperature. Following a further wash, 100 μ l of a sheep anti-complement C1q peroxidase conjugated antibody may be added to each well and incubated for 1 hour at room temperature. The plate may again be washed with wash buffer and 100 μ l of substrate buffer containing OPD (O-phenylenediamine dihydrochloride (Sigma)) may be added to each well. The oxidation reaction, observed by the appearance of a yellow color, may be allowed to proceed for 30 minutes and stopped by the addition of 100 μ l of 4.5 N H₂SO₄. The absorbance may then read at (492-405) nm.

An exemplary polypeptide variant is one that displays a "significant reduction in C1q binding" in this assay. This means that about 100 μ g/ml of the polypeptide variant displays about 50 fold or more reduction in C1q binding compared to 100 μ g/ml of a control antibody having a nonmutated IgG1 Fc region. In the most preferred embodiment, the polypeptide variant "does not bind C1q", *i.e.* 100 μ g/ml of the polypeptide variant displays about 100 fold or more reduction in C1q binding compared to 100 μ g/ml of the control antibody.

Another exemplary variant is one which "has a better binding affinity for human C1q than the parent polypeptide". Such a molecule may display, for example, about two-fold or more, and preferably about five-fold or more, improvement in human C1q binding compared to the parent polypeptide (*e.g.* at the IC₅₀ values for these two molecules). For example, human C1q binding may be about two-fold to about 500-fold, and preferably from about two-fold or from about five-fold to about 1000-fold improved compared to the parent polypeptide.

To assess complement activation, a complement dependent cytotoxicity (CDC) assay may be performed, *e.g.* as described in Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163 (1996). Briefly, various concentrations of the polypeptide variant and human complement may be diluted with buffer. Cells which express the antigen to which the polypeptide variant binds may be diluted to a density of $\sim 1 \times 10^6$ cells /ml. Mixtures of polypeptide variant, diluted human complement and cells expressing the antigen may be added to a flat bottom tissue culture 96 well plate and allowed to incubate for 2 hrs at 37°C and 5% CO₂ to facilitate complement mediated cell lysis. 50 μ l of alamar blue (Accumed International) may then be added to each well and incubated overnight at 37°C. The absorbance is measured using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. The results may be expressed in relative fluorescence units (RFU). The sample concentrations may be computed from a standard curve and the percent activity as compared to nonvariant polypeptide is reported for the polypeptide variant of interest.

Yet another exemplary variant "does not activate complement". For example, 0.6 μ g/ml of the polypeptide variant displays about 0-10% CDC activity in this assay compared to a 0.6

µg/ml of a control antibody having a nonmutated IgG1 Fc region. Preferably the variant does not appear to have any CDC activity in the above CDC assay.

The invention also pertains to a polypeptide variant with enhanced CDC compared to a parent polypeptide, *e.g.*, displaying about two-fold to about 100-fold improvement in CDC activity *in vitro* or *in vivo* (*e.g.* at the IC₅₀ values for each molecule being compared).

A. Receptor Binding Assay and Immune Complex

A receptor binding assay has been developed herein which is particularly useful for determining binding of an analyte of interest to a receptor where the affinity of the analyte for the receptor is relatively weak, *e.g.* in the micromolar range as is the case for FcγRIIA, FcγRIIB, FcγRIIIA and FcγRIIIB. The method involves the formation of a molecular complex that has an improved avidity for the receptor of interest compared to the noncomplexed analyte. The preferred molecular complex is an immune complex comprising: (a) an Fc region-containing polypeptide (such as an antibody or an immunoadhesin); (b) a first target molecule which comprises at least two binding sites for the Fc region-containing polypeptide; and (c) a second target molecule which comprises at least two binding sites for the first target molecule.

In Example 1 below, the Fc region-containing polypeptide is an anti-IgE antibody, such as the E27 antibody (Figs. 4A-4B). E27, when mixed with human IgE at an 1:1 molar ratio, forms a stable hexamer consisting of three E27 molecules and three IgE molecules. In Example 1 below, the "first target molecule" is a chimeric form of IgE in which the Fab portion of an anti-VEGF antibody is fused to the human IgE Fc portion and the "second target molecule" is the antigen to which the Fab binds (*i.e.* VEGF). Each molecule of IgE binds two molecules of VEGF. VEGF also binds two molecules of IgE per molecule of VEGF. When recombinant human VEGF was added at a 2:1 molar ratio to IgE:E27 hexamers, the hexamers were linked into larger molecular weight complexes via the IgE:VEGF interaction (Fig. 5). The Fc region of the anti-IgE antibody of the resultant immune complex binds to FcR with higher avidity than either uncomplexed anti-IgE or anti-IgE:IgE hexamers.

Other forms of molecular complexes for use in the receptor assay are contemplated. Examples comprising only an Fc region-containing polypeptide:first target molecule combination include an immunoadhesin:ligand combination such as VEGF receptor (KDR)-immunoadhesin:VEGF and a full-length bispecific antibody (bsAb):first target molecule. A further example of an Fc region-containing polypeptide:first target molecule:second target molecule combination include a nonblocking antibody:soluble receptor:ligand combination such as anti-Trk antibody:soluble Trk receptor:neurotrophin (Urfer *et al. J. Biol. Chem.* 273(10):5829-5840 (1998)).

Aside from use in a receptor binding assay, the immune complexes described above

have further uses including evaluation of Fc region-containing polypeptide function and immune complex clearance *in vivo*. Hence, the immune complex may be administered to a mammal (*e.g.* in a pre-clinical animal study) and evaluated for its half-life etc.

To determine receptor binding, a polypeptide comprising at least the binding domain
5 of the receptor of interest (*e.g.* the extracellular domain of an α subunit of an FcR) may be coated on solid phase, such as an assay plate. The binding domain of the receptor alone or a receptor-fusion protein may be coated on the plate using standard procedures. Examples of receptor-fusion proteins include receptor-glutathione S-transferase (GST) fusion protein, receptor-chitin binding domain fusion protein, receptor-hexaHis tag fusion protein (coated on
10 glutathione, chitin, and nickel coated plates, respectively). Alternatively, a capture molecule may be coated on the assay plate and used to bind the receptor-fusion protein via the non-receptor portion of the fusion protein. Examples include anti-hexaHis F(ab')₂ coated on the assay plate used to capture receptor-hexaHis tail fusion or anti-GST antibody coated on the assay plate used to capture a receptor-GST fusion. In other embodiments, binding to cells expressing at
15 least the binding domain of the receptor may be evaluated. The cells may be naturally occurring hematopoietic cells that express the FcR of interest or may be transformed with nucleic acid encoding the FcR or a binding domain thereof such that the binding domain is expressed at the surface of the cell to be tested.

The immune complex described hereinabove is added to the receptor-coated plates and
20 incubated for a sufficient period of time such that the analyte binds to the receptor. Plates may then be washed to remove unbound complexes, and binding of the analyte may be detected according to known methods. For example, binding may be detected using a reagent (*e.g.* an antibody or fragment thereof) which binds specifically to the analyte, and which is optionally conjugated with a detectable label (detectable labels and methods for conjugating them to
25 polypeptides are described below in the section entitled "Non-Therapeutic Uses for the Polypeptide Variant").

As a matter of convenience, the reagents can be provided in an assay kit, *i.e.*, a packaged combination of reagents, for combination with the analyte in assaying the ability of the analyte to bind to a receptor of interest. The components of the kit will generally be provided in
30 predetermined ratios. The kit may provide the first target molecule and/or the second target molecule, optionally complexed together. The kit may further include assay plates coated with the receptor or a binding domain thereof (*e.g.* the extracellular domain of the α subunit of an FcR). Usually, other reagents, such as an antibody that binds specifically to the analyte to be assayed, labeled directly or indirectly with an enzymatic label, will also be provided in the kit.
35 Where the detectable label is an enzyme, the kit will include substrates and cofactors required

by the enzyme (*e.g.* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.* assay and/or wash lysis buffer) and the like. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents that substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients that on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the assay.

B. Antibody Preparation

In the preferred embodiment of the invention, the Fc region-containing polypeptide which is modified according to the teachings herein is an antibody. Techniques for producing antibodies follow:

(i) Antigen selection and preparation

Where the polypeptide is an antibody, it is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see US Patent 5,091,178) are also contemplated.

Where the antigen is a polypeptide, it may be a transmembrane molecule (*e.g.* receptor) or ligand such as a growth factor. Exemplary antigens include molecules such as renin; a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor (TF), and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- β ; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such

as TGF- α and TGF- β , including TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, or TGF- β 5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19 and CD20; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon- α , - β , and - γ ; colony stimulating factors (CSFs), *e.g.*, M-CSF, GM-CSF, and G-CSF; interleukins (ILs), *e.g.*, IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

Preferred molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20 and CD34; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150.95, VLA-4, ICAM-1, VCAM, α 4/ β 7 integrin, and α v/ β 3 integrin including either α or β subunits thereof (*e.g.* anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; tissue factor (TF); alpha interferon (α -IFN); an interleukin, such as IL-8; IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; *mpl* receptor; CTLA-4; protein C etc.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (*e.g.* the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (*e.g.* cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule. Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

(ii) *Polyclonal antibodies*

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the relevant antigen to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, SOCl_2 , or $\text{R}^1\text{N}=\text{C}=\text{NR}$, where R and R^1 are

different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 µg or 5 µg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of peptide or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent.

Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

(iii) *Monoclonal antibodies*

Monoclonal antibodies may be made using the hybridoma method first described by Kohler *et al.*, *Nature*, 256:495 (1975), or may be made by recombinant DNA methods (U.S. Patent No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, California USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Maryland USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.*, 133:3001 (1984); Brodeur *et*

al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. Recombinant production of antibodies will be described in more detail below.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison, *et al.*, *Proc. Natl Acad. Sci. USA*, 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

(iv) *Humanized and human antibodies*

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.*, *Nature*, 321:522-525 (1986); Riechmann *et al.*, *Nature*, 332:323-327 (1988); Verhoeven *et al.*, *Science*, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for the humanized antibody (Sims *et al.*, *J. Immunol.*, 151:2296 (1993); Chothia *et al.*, *J. Mol. Biol.*, 196:901 (1987)).

Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:4285 (1992); Presta *et al.*, *J. Immunol.*, 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences

and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, *i.e.*, the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (*e.g.*, mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, *e.g.*, Jakobovits *et al.*, *Proc. Natl. Acad. Sci. USA*, 90:2551 (1993); Jakobovits *et al.*, *Nature*, 362:255-258 (1993); Bruggermann *et al.*, *Year in Immuno.*, 7:33 (1993); and Duchosal *et al.* *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom *et al.*, *J. Mol. Biol.*, 227:381 (1991); Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991); Vaughan *et al.* *Nature Biotech* 14:309 (1996)).

(v) *Multispecific antibodies*

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (*i.e.* bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein. Examples of BsAbs include those with one arm directed against a tumor cell antigen and the other arm directed against a cytotoxic trigger molecule such as anti-Fc γ RI/anti-CD15, anti-p185^{HER2}/Fc γ RIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-CD3/anti-p185^{HER2}, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen (AMOC-31)/anti-CD3; BsAbs with one arm which binds specifically to a tumor antigen and one arm which binds to a toxin such as anti-saporin/anti-Id-1,

anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon- α (IFN- α)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid; BsAbs for converting enzyme activated prodrugs such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol); BsAbs which can be used as fibrinolytic agents such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA); BsAbs for targeting immune complexes to cell surface receptors such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. Fc γ RI, Fc γ RII or Fc γ RIII); BsAbs for use in therapy of infectious diseases such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-Fc γ R/anti-HIV; BsAbs for tumor detection *in vitro* or *in vivo* such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185^{HER2}/anti-hapten; BsAbs as vaccine adjuvants; and BsAbs as diagnostic tools such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti- β -galactosidase. Examples of trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37. Bispecific antibodies can be prepared as full length antibodies or antibody fragments (e.g. F(ab')₂ bispecific antibodies).

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein *et al.*, *Nature*, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker *et al.*, *EMBO J.*, 10:3655-3659 (1991).

According to a different approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light chain binding, present in at least one of the fusions. DNAs encoding the immunoglobulin heavy chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however,

possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance.

In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy chain-light chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690. For further details of generating bispecific antibodies see, for example, Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986). According to another approach described in W096/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first antibody molecule are replaced with larger side chains (*e.g.* tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (*e.g.* alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (US Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in US Patent No. 4,676,980, along with a number of cross-linking techniques.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt *et al.* *J. Immunol.* 147: 60 (1991).

While the polypeptide of interest herein is preferably an antibody, other Fc region-containing polypeptides which can be modified according to the methods described herein are contemplated. An example of such a molecule is an immunoadhesin.

C. Immunoadhesin Preparation

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (*e.g.* the extracellular domain (ECD) of a receptor) with the Fc region of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the

present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc region of immunoglobulin G₁ (IgG₁). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (*i.e.* residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

- (a) AC_L-AC_L;
- (b) AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);
- (c) AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)
- (d) AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);
- (e) V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and
- (f) (A-Y)_n-(V_LC_L-V_HC_H)₂,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;
 C_H is an immunoglobulin heavy chain constant domain;
 n is an integer greater than 1;
 Y designates the residue of a covalent cross-linking agent.

5 In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

10 Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_{H2} domain, or between the C_{H2} and C_{H3} domains. Similar constructs have been reported by Hoogenboom, *et al.*, *Mol. Immunol.* 28:1027-1037 (1991).

15 Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion
20 protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Patent No. 4,816,567, issued 28 March 1989.

25 Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, *e.g.* Aruffo *et al.*, *Cell* 61:1303-1313 (1990); and Stamenkovic *et al.*, *Cell* 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries
30 derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

D. Vectors, Host Cells and Recombinant Methods

The invention also provides isolated nucleic acid encoding a polypeptide variant as disclosed herein, vectors and host cells comprising the nucleic acid, and recombinant techniques for the production of the polypeptide variant.

5 For recombinant production of the polypeptide variant, the nucleic acid encoding it is isolated and inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. DNA encoding the polypeptide variant is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the polypeptide variant). Many vectors are available. The vector
10 components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence.

(i) Signal sequence component

The polypeptide variant of this invention may be produced recombinantly not only
15 directly, but also as a fusion polypeptide with a heterologous polypeptide, which is preferably a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. The heterologous signal sequence selected preferably is one that is recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell. For prokaryotic host cells that do not recognize and process the native polypeptide variant signal
20 sequence, the signal sequence is substituted by a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the native signal sequence may be substituted by, *e.g.*, the yeast invertase leader, α factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders), or acid phosphatase leader, the *C. albicans* glucoamylase leader, or the signal
25 described in WO 90/13646. In mammalian cell expression, mammalian signal sequences as well as viral secretory leaders, for example, the herpes simplex gD signal, are available.

The DNA for such precursor region is ligated in reading frame to DNA encoding the polypeptide variant.

(ii) Origin of replication component

30 Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication
35 from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 μ plasmid origin is

suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used only because it contains the early promoter).

5 (iii) Selection gene component

Expression and cloning vectors may contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene
10 encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin, mycophenolic acid and hygromycin.

15 Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the polypeptide variant nucleic acid, such as DHFR, thymidine kinase, metallothionein-I and -II, preferably primate metallothionein genes, adenosine deaminase, ornithine decarboxylase, etc.

For example, cells transformed with the DHFR selection gene are first identified by
20 culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity.

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding polypeptide variant, wild-type
25 DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid
30 YRp7 (Stinchcomb *et al.*, *Nature*, 282:39 (1979)). The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1. Jones, *Genetics*, 85:12 (1977). The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626)
35 are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for

transformation of *Kluyveromyces* yeasts. Alternatively, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis*. Van den Berg, *Bio/Technology*, 8:135 (1990). Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed. Fleer *et al.*, *Bio/Technology*, 9:968-975 (1991).

(iv) Promoter component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the polypeptide variant nucleic acid. Promoters suitable for use with prokaryotic hosts include the *phoA* promoter, β -lactamase and lactose promoter systems, alkaline phosphatase, a tryptophan (*trp*) promoter system, and hybrid promoters such as the *tac* promoter. However, other known bacterial promoters are suitable. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding the polypeptide variant.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CNCAAT region where N may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase or other glycolytic enzymes, such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Polypeptide variant transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from

heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978. See also Reyes *et al.*, *Nature* 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus. Alternatively, the rous sarcoma virus long terminal repeat can be used as the promoter.

(v) *Enhancer element component*

Transcription of a DNA encoding the polypeptide variant of this invention by higher eukaryotes is often increased by inserting an enhancer sequence into the vector. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, *Nature* 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the polypeptide variant-encoding sequence, but is preferably located at a site 5' from the promoter.

(vi) *Transcription termination component*

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding the polypeptide variant. One useful transcription termination component is the bovine growth hormone polyadenylation region. See WO94/11026 and the expression vector disclosed therein.

(vii) *Selection and transformation of host cells*

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example,

Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*.

- 5 One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, *E. coli* X1776 (ATCC 31,537), and *E. coli* W3110 (ATCC 27,325) are suitable. These examples are illustrative rather than limiting.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for polypeptide variant-encoding vectors. *Saccharomyces*
 10 *cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomyces pombe*; *Kluyveromyces* hosts such as, e.g., *K. lactis*, *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickerhamii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilum* (ATCC 36,906), *K. thermotolerans*, and *K.*
 15 *marxianus*; *yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070); *Candida*; *Trichoderma reesia* (EP 244,234); *Neurospora crassa*; *Schwanniomyces* such as *Schwanniomyces occidentalis*; and filamentous fungi such as, e.g., *Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

Suitable host cells for the expression of glycosylated polypeptide variant are derived from
 20 multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV
 25 and the Bm-5 strain of *Bombyx mori* NPV, and such viruses may be used as the virus herein according to the present invention, particularly for transfection of *Spodoptera frugiperda* cells.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can also be utilized as hosts.

However, interest has been greatest in vertebrate cells, and propagation of vertebrate
 30 cells in culture (tissue culture) has become a routine procedure. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al.*, *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub *et al.*, *Proc. Natl. Acad. Sci. USA* 77:4216
 35 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587);

human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; and
5 a human hepatoma line (Hep G2).

Host cells are transformed with the above-described expression or cloning vectors for polypeptide variant production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

10 (viii) *Culturing the host cells*

The host cells used to produce the polypeptide variant of this invention may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), (Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media
15 described in Ham *et al.*, *Meth. Enz.* 58:44 (1979), Barnes *et al.*, *Anal. Biochem.* 102:255 (1980), U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO 90/03430; WO 87/00195; or U.S. Patent Re. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium,
20 magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture
25 conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

(ix) *Polypeptide variant purification*

When using recombinant techniques, the polypeptide variant can be produced intracellularly, in the periplasmic space, or directly secreted into the medium. If the polypeptide
30 variant is produced intracellularly, as a first step, the particulate debris, either host cells or lysed fragments, is removed, for example, by centrifugation or ultrafiltration. Carter *et al.*, *Bio/Technology* 10:163-167 (1992) describe a procedure for isolating antibodies which are secreted to the periplasmic space of *E. coli*. Briefly, cell paste is thawed in the presence of sodium acetate (pH 3.5), EDTA, and phenylmethylsulfonylfluoride (PMSF) over about 30 min.
35 Cell debris can be removed by centrifugation. Where the polypeptide variant is secreted into the medium, supernatants from such expression systems are generally first concentrated using a

commercially available protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. A protease inhibitor such as PMSF may be included in any of the foregoing steps to inhibit proteolysis and antibiotics may be included to prevent the growth of adventitious contaminants.

5 The polypeptide variant composition prepared from the cells can be purified using, for example, hydroxylapatite chromatography, gel electrophoresis, dialysis, and affinity chromatography, with affinity chromatography being the preferred purification technique. The suitability of protein A as an affinity ligand depends on the species and isotype of any immunoglobulin Fc region that is present in the polypeptide variant. Protein A can be used to
10 purify polypeptide variants that are based on human $\gamma 1$, $\gamma 2$, or $\gamma 4$ heavy chains (Lindmark *et al.*, *J. Immunol. Meth.* 62:1-13 (1983)). Protein G is recommended for all mouse isotypes and for human $\gamma 3$ (Guss *et al.*, *EMBO J.* 5:15671575 (1986)). The matrix to which the affinity ligand is attached is most often agarose, but other matrices are available. Mechanically stable matrices such as controlled pore glass or poly(styrenedivinyl)benzene allow for faster flow rates and
15 shorter processing times than can be achieved with agarose. Where the polypeptide variant comprises a C_H3 domain, the Bakerbond ABXTM resin (J. T. Baker, Phillipsburg, NJ) is useful for purification. Other techniques for protein purification such as fractionation on an ion-exchange column, ethanol precipitation, Reverse Phase HPLC, chromatography on silica, chromatography on heparin SEPHAROSETM chromatography on an anion or cation exchange resin (such as a
20 polyaspartic acid column), chromatofocusing, SDS-PAGE, and ammonium sulfate precipitation are also available depending on the polypeptide variant to be recovered.

 Following any preliminary purification step(s), the mixture comprising the polypeptide variant of interest and contaminants may be subjected to low pH hydrophobic interaction chromatography using an elution buffer at a pH between about 2.5-4.5, preferably performed at
25 low salt concentrations (*e.g.*, from about 0-0.25M salt).

E. Pharmaceutical Formulations

 Therapeutic formulations of the polypeptide variant are prepared for storage by mixing the polypeptide variant having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (*Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable
30 carriers, excipients, or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride;
35 phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol;

resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptide; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g., Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsule prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsule and poly-(methylmethacrylate) microcapsule, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nanoparticles and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition, Osol, A. Ed. (1980).

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the polypeptide variant, which matrices are in the form of shaped articles, e.g., films, or microcapsule. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)), polylactides (U.S. Pat. No. 3,773,919), copolymers of L-glutamic acid and γ ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanism involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thio-disulfide

interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

5 **F. Non-Therapeutic Uses for the Polypeptide Variant**

The polypeptide variant of the invention may be used as an affinity purification agent. In this process, the polypeptide variant is immobilized on a solid phase such a Sephadex resin or filter paper, using methods well known in the art. The immobilized polypeptide variant is contacted with a sample containing the antigen to be purified, and thereafter the support is
10 washed with a suitable solvent that will remove substantially all the material in the sample except the antigen to be purified, which is bound to the immobilized polypeptide variant. Finally, the support is washed with another suitable solvent, such as glycine buffer, pH 5.0, that will release the antigen from the polypeptide variant.

The polypeptide variant may also be useful in diagnostic assays, *e.g.*, for detecting
15 expression of an antigen of interest in specific cells, tissues, or serum.

For diagnostic applications, the polypeptide variant typically will be labeled with a detectable moiety. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as ^{35}S , ^{14}C , ^{125}I , ^3H , and ^{131}I . The polypeptide variant can
20 be labeled with the radioisotope using the techniques described in *Current Protocols in Immunology*, Volumes 1 and 2, Coligen *et al.*, Ed. Wiley-Interscience, New York, New York, Pubs. (1991) for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas
25 Red are available. The fluorescent labels can be conjugated to the polypeptide variant using the techniques disclosed in *Current Protocols in Immunology*, *supra*, for example. Fluorescence can be quantified using a fluorimeter.

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyzes a chemical alteration of
30 the chromogenic substrate that can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical
35 reaction and may then emit light which can be measured (using a chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include

luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase, β -galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in *Methods in Enzym.* (ed J. Langone & H. Van Vunakis), Academic press, New York, 73:147-166 (1981).

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxide as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.*, orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB));

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate; and

(iii) β -D-galactosidase (β -D-Gal) with a chromogenic substrate (*e.g.*, p-nitrophenyl- β -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- β -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the polypeptide variant. The skilled artisan will be aware of various techniques for achieving this. For example, the polypeptide variant can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the polypeptide variant in this indirect manner. Alternatively, to achieve indirect conjugation of the label with the polypeptide variant, the polypeptide variant is conjugated with a small hapten (*e.g.*, digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten polypeptide variant (*e.g.*, anti-digoxin antibody). Thus, indirect conjugation of the label with the polypeptide variant can be achieved.

In another embodiment of the invention, the polypeptide variant need not be labeled, and the presence thereof can be detected using a labeled antibody which binds to the polypeptide variant.

The polypeptide variant of the present invention may be employed in any known assay method, such as competitive binding assays, direct and indirect sandwich assays, and immunoprecipitation assays. Zola, *Monoclonal Antibodies: A Manual of Techniques*, pp.147-158 (CRC Press, Inc. 1987).

The polypeptide variant may also be used for *in vivo* diagnostic assays. Generally, the polypeptide variant is labeled with a radionuclide (such as ^{111}In , ^{99}Tc , ^{14}C , ^{131}I , ^{125}I , ^3H , ^{32}P or ^{35}S) so that the antigen or cells expressing it can be localized using immunoscintigraphy.

G. *In Vivo* Uses for the Polypeptide Variant

It is contemplated that the polypeptide variant of the present invention may be used to treat a mammal *e.g.* a patient suffering from, or predisposed to, a disease or disorder who could benefit from administration of the polypeptide variant. The conditions which can be treated with the polypeptide variant are many and include cancer (*e.g.* where the polypeptide variant binds the HER2 receptor, CD20 or vascular endothelial growth factor (VEGF)); allergic conditions such as asthma (with an anti-IgE antibody); and LFA-1-mediated disorders (*e.g.* where the polypeptide variant is an anti-LFA-1 or anti-ICAM-1 antibody) etc.

Where the antibody binds the HER2 receptor, the disorder preferably is HER2-expressing cancer, *e.g.* a benign or malignant tumor characterized by overexpression of the HER2 receptor. Such cancers include, but are not limited to, breast cancer, squamous cell cancer, small-cell lung cancer, non-small cell lung cancer, gastrointestinal cancer, pancreatic cancer, glioblastoma, cervical cancer, ovarian cancer, bladder cancer, hepatoma, colon cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, liver cancer, prostate cancer, vulval cancer, thyroid cancer, hepatic carcinoma and various types of head and neck cancer. According to the teachings herein, one may prepare a polypeptide with a variant Fc region which has improved, or diminished, ADCC activity. Such molecules will find applications in the treatment of different disorders.

For example, the polypeptide variant with improved ADCC activity may be employed in the treatment of diseases or disorders where destruction or elimination of tissue or foreign micro-organisms is desired. For example, the polypeptide may be used to treat cancer; inflammatory disorders; infections (*e.g.* bacterial, viral, fungal or yeast infections); and other conditions (such as goiter) where removal of tissue is desired, etc.

Where the polypeptide variant has diminished ADCC activity, such variants may be used to treat diseases or disorders where a Fc region-containing polypeptide with long half-life is desired, but the polypeptide preferably does not have undesirable effector function(s). For example, the Fc region-containing polypeptide may be an anti-tissue factor (TF) antibody; anti-IgE antibody; and anti-integrin antibody (*e.g.* an anti- $\alpha 4\beta 7$ antibody). The desired mechanism of action of such Fc region-containing polypeptides may be to block ligand-receptor binding pairs. Moreover, the Fc-region containing polypeptide with diminished ADCC activity may be an agonist antibody.

The polypeptide variant is administered by any suitable means, including parenteral, subcutaneous, intraperitoneal, intrapulmonary, and intranasal, and, if desired for local

immunosuppressive treatment, intralesional administration. Parenteral infusions include intramuscular, intravenous, intraarterial, intraperitoneal, or subcutaneous administration. In addition, the polypeptide variant is suitably administered by pulse infusion, particularly with declining doses of the polypeptide variant. Preferably the dosing is given by injections, most
5 preferably intravenous or subcutaneous injections, depending in part on whether the administration is brief or chronic.

For the prevention or treatment of disease, the appropriate dosage of polypeptide variant will depend on the type of disease to be treated, the severity and course of the disease, whether the polypeptide variant is administered for preventive or therapeutic purposes, previous
10 therapy, the patient's clinical history and response to the polypeptide variant, and the discretion of the attending physician. The polypeptide variant is suitably administered to the patient at one time or over a series of treatments.

Depending on the type and severity of the disease, about 1 $\mu\text{g/kg}$ to 15 mg/kg (e.g., 0.1-20 mg/kg) of polypeptide variant is an initial candidate dosage for administration to the patient, whether, for example, by one or more separate administrations, or by continuous infusion. A
15 typical daily dosage might range from about 1 $\mu\text{g/kg}$ to 100 mg/kg or more, depending on the factors mentioned above. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily
20 monitored by conventional techniques and assays.

The polypeptide variant composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method
25 of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of the polypeptide variant to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat a disease or disorder. The polypeptide variant need not be, but is optionally formulated with one or more agents currently used to prevent or treat the disorder
30 in question. The effective amount of such other agents depends on the amount of polypeptide variant present in the formulation, the type of disorder or treatment, and other factors discussed above. These are generally used in the same dosages and with administration routes as used hereinbefore or about from 1 to 99% of the heretofore employed dosages.

The invention will be more fully understood by reference to the following examples. They
35 should not, however, be construed as limiting the scope of this invention. All literature and

patent citations mentioned herein are expressly incorporated by reference.

EXAMPLE 1

Low Affinity Receptor Binding Assay

5 This assay determines binding of an IgG Fc region to recombinant Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA α subunits expressed as His6-glutathione S transferase (GST)-tagged fusion proteins. Since the affinity of the Fc region of IgG1 for the Fc γ RI is in the nanomolar range, the binding of IgG1 Fc variants can be measured by titrating monomeric IgG and measuring bound IgG with a polyclonal anti-IgG in a standard ELISA format (Example 2 below). The affinity of the other
10 members of the Fc γ R family, *i.e.* Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA for IgG is however in the micromolar range and binding of monomeric IgG1 for these receptors can not be reliably measured in an ELISA format.

The following assay utilizes Fc variants of recombinant anti-IgE E27 (Figures 4A and 4B) which, when mixed with human IgE at a 1:1 molar ratio, forms a stable hexamer consisting of
15 three anti-IgE molecules and three IgE molecules. A recombinant chimeric form of IgE (chimeric IgE) was engineered and consists of a human IgE Fc region and the Fab of an anti-VEGF antibody (Presta *et al. Cancer Research* 57:4593-4599 (1997)) which binds two VEGF molecules per mole of anti-VEGF. When recombinant human VEGF is added at a 2:1 molar ratio to chimeric IgE:E27 hexamers, the hexamers are linked into larger molecular weight complexes
20 via the chimeric IgE Fab:VEGF interaction. The E27 component of this complex binds to the Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA α subunits with higher avidity to permit detection in an ELISA format.

MATERIALS AND METHODS

Receptor Coat: Fc γ receptor α subunits were expressed as GST fusions of His6 tagged
25 extracellular domains (ECDs) in 293 cells resulting in an ECD-6His-GST fusion protein (Graham *et al. J. Gen. Virol.* 36:59-74 (1977) and Gorman *et al. DNA Prot. Eng. Tech.* 2:3-10 (1990)) and purified by Ni-NTA column chromatography (Qiagen, Australia) and buffer exchanged into phosphate buffered saline (PBS). Concentrations were determined by absorption at 280nm using extinction coefficients derived by amino acid composition analysis. Receptors were coated
30 onto Nunc F96 maxisorb plates (cat no. 439454) at 100ng per well by adding 100 μ l of receptor-GST fusion at 1 μ g/ml in PBS and incubated for 48 hours at 4°C. Prior to assay, plates are washed 3x with 250 μ l of wash buffer (PBS pH 7.4 containing 0.5% TWEEN 20™) and blocked with 250 μ l of assay buffer (50mM Tris buffered saline, 0.05% TWEEN 20™, 0.5% RIA grade bovine albumin (Sigma A7888), and 2mM EDTA pH 7.4).

35 **Immune Complex Formation:** Equal molar amounts (1:1) of E27 and recombinant

chimeric IgE which binds two moles recombinant human VEGF per mole of chimeric IgE are added to a 12 x 75mm polypropylene tube in PBS and mixed by rotation for 30 minutes at 25°C.

E27 (anti-IgE) /chimeric IgE (IgE) hexamers are formed during this incubation. Recombinant human VEGF (165 form, MW 44,000) is added at a 2:1 molar ratio to the IgE concentration and mixed by rotation an additional 30 minutes at 25°C. VEGF-chimeric IgE binding links E27:chimeric IgE hexamers into larger molecular weight complexes which bind FcγR α subunit ECD coated plates via the Fc region of the E27 antibody.

E27:chimeric IgE:VEGF: (1:1:2 molar ratio) complexes are added to FcγR α subunit coated plates at E27 concentrations of 5 μg and 1 μg total IgG in quadruplicate in assay buffer and incubated for 120 minutes at 25°C on an orbital shaker.

Complex Detection: Plates are washed 5x with wash buffer to remove unbound complexes and IgG binding is detected by adding 100 μl horse radish peroxidase (HRP) conjugated goat anti-human IgG (γ) heavy chain specific (Boehringer Mannheim 1814249) at 1:10,000 in assay buffer and incubated for 90min at 25°C on an orbital shaker. Plates are washed 5x with wash buffer to remove unbound HRP goat anti-human IgG and bound anti-IgG is detected by adding 100 μl of substrate solution (0.4mg/ml o-phenylenediamine dihydrochloride, Sigma P6912, 6 mM H₂O₂ in PBS) and incubating for 8 min at 25°C. Enzymatic reaction is stopped by the addition of 100 μl 4.5N H₂SO₄ and colorimetric product is measured at 490 nm on a 96 well plate densitometer (Molecular Devices). Binding of E27 variant complexes is expressed as a percent of the wild type E27 containing complex.

EXAMPLE 2

Identification of Unique C1q Binding Sites in a Human IgG Antibody

In the present study, mutations were identified in the CH2 domain of a human IgG1 antibody, "C2B8" (Reff *et al.*, *Blood* 83:435 (1994)), that ablated binding of the antibody to C1q but did not alter the conformation of the antibody nor affect binding to each of the FcγRs. By alanine scanning mutagenesis, five variants in human IgG1 were identified, D270K, D270V, K322A P329A, and P331, that were non-lytic and had decreased binding to C1q. The data suggested that the core C1q binding sites in human IgG1 is different from that of murine IgG2b.

In addition, K322A, P329A and P331A were found to bind normally to the CD20 antigen, and to four Fc receptors, FcγRI, FcγRII, FcγRIII and FcRn.

MATERIALS AND METHODS

Construction of C2B8 Variants: The chimeric light and heavy chains of anti-CD20 antibody C2B8 (Reff *et al.*, *Blood* 83:435 (1994)) subcloned separately into previously described PRK vectors (Gorman *et al.*, *DNA Protein Eng. Tech.* 2:3 (1990)) were used. By site directed

mutagenesis (Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488 (1987)), alanine scan variants of the Fc region in the heavy chain were constructed. The heavy and light chain plasmids were co-transfected into an adenovirus transformed human embryonic kidney cell line as previously described (Werther *et al.*, *J. Immunol.* 157:4986 (1996)). The media was changed to serum-free
5 24 hours after transfection and the secreted antibody was harvested after five days. The antibodies were purified using Protein A-SEPHAROSE CL-4B™ (Pharmacia), buffer exchanged and concentrated to 0.5 ml with PBS using a Centricon-30 (Amicon), and stored at 4°C. The concentration of the antibody was determined using total Ig-binding ELISA.

C1q Binding ELISA: Costar 96 well plates were coated overnight at 4°C with the
10 indicated concentrations of C2B8 in coating buffer (0.05 M sodium carbonate buffer), pH 9. The plates were then washed 3x with PBS/ 0.05% TWEEN 20™, pH 7.4 and blocked with 200µl of ELISA diluent without thimerosal (0.1M NaPO₄ / 0.1M NaCl / 0.1% gelatin / 0.05% TWEEN 20™/ 0.05% ProClin300) for 1hr at room temperature. The plate was washed 3x with wash buffer, an aliquot of 100µl of 2 µg/ml C1q (Quidel, San Diego, CA) was added to each well and incubated
15 for 2 hrs at room temperature. The plate was then washed 6x with wash buffer. 100 µl of a 1:1000 dilution of sheep anti-complement C1q peroxidase conjugated antibody (Biodesign) was added to each well and incubated for 1 hour at room temperature. The plate was again washed 6x with wash buffer and 100 µl of substrate buffer (PBS/0.012% H₂O₂) containing OPD (O-phenylenediamine dihydrochloride (Sigma)) was added to each well. The oxidation reaction,
20 observed by the appearance of a yellow color, was allowed to proceed for 30 minutes and stopped by the addition of 100 µl of 4.5 N H₂SO₄. The absorbance was then read at (492-405) nm using a microplate reader (SPECTRA MAX 250™, Molecular Devices Corp.). The appropriate controls were run in parallel (*i.e.* the ELISA was performed without C1q for each concentration of C2B8 used and also the ELISA was performed without C2B8). For each
25 variant, C1q binding was measured by plotting the absorbance (492-405) nm versus concentration of C2B8 in µg/ml using a 4-parameter curve fitting program (KALEIDAGRAPH™) and comparing EC₅₀ values.

Complement Dependent Cytotoxicity (CDC) Assay: This assay was performed essentially as previously described (Gazzano-Santoro *et al.*, *J. Immunol. Methods* 202:163
30 (1996)). Various concentrations of C2B8 (0.08-20 µg/ml) were diluted with RHB buffer (RPMI 1640/20mM HEPES (pH 7.2)/2mM Glutamine/0.1% BSA/100 µg/ml Gentamicin). Human complement (Quidel) was diluted 1:3 in RHB buffer and WIL2-S cells (available from the ATCC, Manassas, VA) which express the CD20 antigen were diluted to a density of 1 x 10⁶ cells /ml with RHB buffer. Mixtures of 150 µl containing equal volumes of C2B8, diluted human
35 complement and WIL2-S cells were added to a flat bottom tissue culture 96 well plate and

allowed to incubate for 2 hrs at 37°C and 5% CO₂ to facilitate complement mediated cell lysis. 50 µl of alamar blue (Accumed International) was then added to each well and incubated overnight at 37°C. The absorbance was measured using a 96-well fluorometer with excitation at 530 nm and emission at 590 nm. As described by Gazzano-Santoro *et al.*, the results are expressed in relative fluorescence units (RFU). The sample concentrations were computed from a C2B8 standard curve and the percent activity as compared to wild type C2B8 is reported for each variant.

CD20 Binding Potency of the C2B8 Variants: The binding of C2B8 and variants to the CD20 antigen were assessed by a method previously described (Reff *et al.*, (1994), *supra*; reviewed in Gazzano-Santoro *et al.*, (1996), *supra*). WIL2-S cells were grown for 3-4 days to a cell density of 1 x 10⁶ cells/ml. The cells were washed and spun twice in FACS buffer (PBS/0.1% BSA/0.02% NaN₃) and resuspended to a cell density of 5 x 10⁶ cells/ml. 200 µl of cells (5 x 10⁶ cells/ml) and 20 µl of diluted C2B8 samples were added to a 5 ml tube and incubated at room temperature for 30 minutes with agitation. The mixture was then washed with 2 ml of cold FACS buffer, spun down and resuspended in 200 µl of cold FACS buffer. To the suspension, 10 µl of goat anti-human IgG-FITC (American Qualex Labs.) was added and the mixture was incubated in the dark at room temperature for 30 minutes with agitation. After incubation, the mixture was washed with 2 ml of FACS buffer, spun down and resuspended in 1 ml of cold fixative buffer (1% formaldehyde in PBS). The samples were analyzed by flow cytometry and the results expressed as relative fluorescence units (RFU) were plotted against antibody concentrations using a 4-parameter curve fitting program (KALEIDAGRAPH™). The EC₅₀ values are reported as a percentage of that of the C2B8 reference material.

FcγR Binding ELISAs: FcγRI α subunit-GST fusion was coated onto Nunc F96 maxisorb plates (cat no. 439454) by adding 100 µl of receptor-GST fusion at 1 µg/ml in PBS and incubated for 48 hours at 4°C. Prior to assay, plates are washed 3x with 250 µl of wash buffer (PBS pH 7.4 containing 0.5% TWEEN 20™) and blocked with 250 µl of assay buffer (50mM Tris buffered saline, 0.05% TWEEN 20™, 0.5% RIA grade bovine albumin (Sigma A7888), and 2mM EDTA pH 7.4). Samples diluted to 10µg/ml in 1 ml of assay buffer are added to FcγRI α subunit coated plates and incubated for 120 minutes at 25°C on an orbital shaker. Plates are washed 5x with wash buffer to remove unbound complexes and IgG binding is detected by adding 100 µl horse radish peroxidase (HRP) conjugated goat anti-human IgG (γ) heavy chain specific (Boehringer Mannheim 1814249) at 1:10,000 in assay buffer and incubated for 90min at 25°C on an orbital shaker. Plates are washed 5x with wash buffer to remove unbound HRP goat anti-human IgG and bound anti-IgG is detected by adding 100 µl of substrate solution (0.4mg/ml o-phenylenedaimine dihydrochloride, Sigma P6912, 6 mM H₂O₂ in PBS) and incubating for 8 min

at 25°C. Enzymatic reaction is stopped by the addition of 100 µl 4.5N H₂SO₄ and colorimetric product is measured at 490 nm on a 96 well plate densitometer (Molecular Devices). Binding of variant is expressed as a percent of the wild type molecule.

FcγRII and III binding ELISAs were performed as described in Example 1 above.

5 For measuring FcRn binding activity of IgG variants, ELISA plates were coated with 2 µg/ml streptavidin (Zymed, South San Francisco) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% BSA, pH 7.2 at room temperature for one hour. Biotinylated FcRn (prepared using biotin-X-NHS from Research Organics, Cleveland, OH and used at 1-2 µg/ml) in PBS-0.5% BSA, 0.05% polysorbate 20, pH 7.2, was added to the plate and
10 incubated for one hour. Two fold serial dilutions of IgG standard (1.6-100 ng/ml) or variants in PBS-0.5% BSA, 0.05% polysorbate 20, pH 6.0, were added to the plate and incubated for two hours. Bound IgG was detected using peroxidase labeled goat F(ab')₂ anti-human IgG F(ab')₂ in the above pH 6.0 buffer (Jackson ImmunoResearch, West Grove, PA) followed by 3,3',5,5'-tetramethyl benzidine (Kirgaard & Perry Laboratories) as the substrate. Plates were washed
15 between steps with PBS-0.05% polysorbate 20 at either pH 7.2 or 6.0. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, CA). Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy software, Reading, PA). Concentrations of IgG variants corresponding to the mid-point absorbance of the titration curve of the standard were calculated and then divided by the
20 concentration of the standard corresponding to the mid-point absorbance of the standard titration curve.

RESULTS AND DISCUSSION

By alanine scanning mutagenesis, several single point mutations were constructed in the
25 CH2 domain of C2B8 beginning with E318A, K320A and K322A. All the variants constructed bound normally to the CD20 antigen (Table 3).

Table 3

	wt	E318A	K320A	K322A	P329A	P331A
FcRn	+	+	+	+		
CD20	+	+	+	+	+	+
FcγRI	+	+	+	+	+	+
FcγRII	+	+	+	+	+	+
FcγRIII	+	+	+	+	+	+
*C1q	+++	++	+++	-	-	-
CDC	+	+	+	-	-	-

(+) indicates binding and (-) signifies binding abolished

*With respect to C1q binding, each + sign is equivalent to approximately 33% binding.

Where binding of human complement to an antibody with a human Fc was analyzed, the ability of E318A and K320A to activate complement was essentially identical to that of wild type C2B8 (Table 3). When compared to wild type C2B8, there appears to be little difference in the binding of E318A and K320A to C1q. There is only a 10% decrease in the binding of K320A and about a 30% decrease in the binding of E318A to C1q (Fig. 2). The results indicate that the effect of the E318A and the K320A substitution on complement activation and C1q binding is minimal. Also, the human IgG1 of C2B8 was substituted for human IgG2 and used as a negative control in the C1q binding studies. The IgG2 variant appears to have a much lower affinity for C1q than the E318A and K320A variants (Fig. 2). Thus, the results demonstrate that E318 and K320 do not constitute the core C1q binding sites for human IgG1. Conversely, the K322A substitution had a significant effect on both complement activity and C1q binding. The K322A variant did not have CDC activity when tested in the above CDC assay and was more than a 100 fold lower than wild type C2B8 in binding to C1q (Fig. 2). In the human system, K322 is the only residue of the proposed core C1q binding sites that appeared to have a significant effect on complement activation and C1q binding.

Since the Duncan and Winter study was performed using mouse IgG2b and the above results reveal that K320 and E318 in human IgG1 are not involved in C1q binding, and without being bound to any one theory, the above data suggest that the C1q binding region in murine IgGs is different from that of the human. To investigate this further and also to identify additional variants that do not bind to C1q and hence do not activate complement, several more point mutations in the vicinity of K322 were constructed as assessed from the three dimensional structure of the C2B8 Fc. Variants constructed, K274A, N276A, Y278A, S324A, P329A, P331A, K334A, and T335A, were assessed for their ability to bind C1q and also to activate complement.

Many of these substitutions had little or no effect on C1q binding or complement activation. In the above assays, the P329A and the P331A variants did not activate complement and had decreased binding to C1q. The P331A variant did not activate complement and was 60 fold lower in binding to C1q (Fig. 3) when compared to wild type C2B8 (Fig. 2). The concentration range of the antibody variants used in Fig. 3 is expanded to 100µg/ml in order to observe saturation of C1q binding to the P331A variant. The mutation P329A results in an antibody that does not activate complement and is more than a 100 fold lower in binding to C1q (Fig. 3) when compared to wild type C2B8 (Fig. 2).

Variants that did not bind to C1q and hence did not activate complement were examined for their ability to bind to the Fc receptors: FcγRI, FcγRIIA, FcγRIIB, FcγRIIIA and FcγRn. This particular study was performed using a humanized anti-IgE antibody, an IgG1 antibody with these mutations (see Example 1 above). The results revealed the variants, K322A and P329A, bind to all the Fc receptors to the same extent as the wild type protein (Table 4). However, there was a slight decrease in the binding of P331A to FcγRIIB.

In conclusion, two amino acid substitutions in the COOH terminal region of the CH2 domain of human IgG1, K322A and P329A were identified that result in more than 100 fold decrease in C1q binding and do not activate the CDC pathway. These two variants, K322A and P329A, bind to all Fc receptors with the same affinity as the wild type antibody. Based on the results, summarized in Table 4, and without being bound to any one theory, it is proposed that the C1q binding epicenter of human IgG1 is centered around K322, P329 and P331 and is different from the murine IgG2b epicenter which constitutes E318, K320 and K322.

Table 4

	wt	E318A	K320A	K322A	P329A	P331A
CD20	100	89	102	86	112	103
^a FcγRI	100	93	102	90	104	74
^a FcγRIIA	100	113	94	109	111	86
^a FcγRIIB	100	106	83	101	96	58
^a FcγRIII	100	104	72	90	85	73
CDC	100	108	108	none	none	none

^a For binding to the FcγRs the variants were made in the E27 background (anti-IgE).

The results are presented as a percentage of the wild type.

A further residue involved in binding human C1q was identified using the methods described in the present example. The residue D270 was replaced with lysine and valine to

generate variants D270K and D270V, respectively. These variants both showed decreased binding to human C1q (Fig. 6) and were non-lytic (Fig. 7). The two variants bound the CD20 antigen normally and recruited ADCC.

5

EXAMPLE 3

Variants with Improved C1q Binding

The following study shows that substitution of residues at positions K326, A327, E333 and K334 resulted in variants with at least about a 30% increase in binding to C1q when compared to the wild type antibody. This indicated K326, A327, E333 and K334 are potential sites for improving the efficacy of antibodies by way of the CDC pathway. The aim of this study was to improve CDC activity of an antibody by increasing binding to C1q. By site directed mutagenesis at K326 and E333, several variants with increased binding to C1q were constructed. The residues in order of increased binding at K326 are K<V<E<A<G<D<M<W, and the residues in order of increased binding at E333 are E<Q<D<V<G<A<S. Four variants, K326M, K326D, K326E and E333S were constructed with at least a two-fold increase in binding to C1q when compared to wild type. Variant K326W displayed about a five-fold increase in binding to C1q.

Variants of the wild type C2B8 antibody were prepared as described above in Example 2. A further control antibody, wild type C2B8 produced in Chinese hamster ovary (CHO) cells essentially as described in US Patent 5,736,137, was included in a C1q binding ELISA to confirm that wt C2B8 produced in the 293 kidney cell line had the same C1q binding activity as the CHO-produced antibody (see "CHO-wt-C2B8" in Fig. 8). The C1q binding ELISA, CDC assay, and CD20 binding potency assay in this example were performed as described in Example 2 above.

As shown in Fig. 8, alanine substitution at K326 and E333 in C2B8 resulted in variants with about a 30% increase in binding to C1q.

Several other single point variants at K326 and E333 were constructed and assessed for their ability to bind C1q and activate complement. All the variants constructed bound normally to the CD20 antigen.

With respect to K326, the other single point variants constructed were K326A, K326D, K326E, K326G, K326V, K326M and K326W. As shown in Fig. 9, these variants all bound to C1q with a better affinity than the wild type antibody. K326W, K326M, K326D and K326E showed at least a two-fold increase in binding to C1q (Table 5). Among the K326 variants, K326W had the best affinity for C1q.

Table 5

Variant	EC ₅₀ value
Wild type	1.53
K326V	1.30
K326A	1.03
K326E	1.08
K326G	0.95
K326D	0.76
K326M	0.67
K326W	0.47
E333S	0.81
E333A	0.98
E333G	1.14
E333V	1.18
E333D	1.22
E333Q	1.52
K334A	1.07

Substitutions with hydrophobic as well as charged residues resulted in variants with increased binding to C1q. Even substitution with glycine which is known to impart flexibility to a chain and is well conserved in nature, resulted in a variant with higher affinity for C1q when compared to the wild type. It would appear that any amino acid substitution at this site would result in a variant with higher affinity for C1q. As assessed from the three-dimensional structure, K326 and E333 are in the vicinity of the core C1q binding sites (Fig. 10).

In addition to alanine, E333 was also substituted with other amino acid residues. These variants, E333S, E333G, E333V, E333D, and E333Q, all had increased binding to C1q when compared to the wild type (Fig. 11). As shown in Table 5, the order of binding affinity for C1q was as follows: E333S>E333A>E333G>E333V>E333D>E333Q. Substitutions with amino acid residues with small side chain volumes, *i.e.* serine, alanine and glycine, resulted in variants with higher affinity for C1q in comparison to the other variants, E333V, E333D and E333Q, with larger side chain volumes. The variant E333S had the highest affinity for C1q, showing a two-fold increase in binding when compared to the wild type. Without being bound to any one theory, this indicates the effect on C1q binding at 333 may also be due in part to the polarity of the residue.

Double variants were also generated. As shown in Figs. 12 and 13, double variants K326M-E333S and K326A-E333A were at least three-fold better at binding human C1q than wild

type C2B8 (Fig. 12) and at least two-fold better at mediating CDC compared to wild type C2B8 (Fig. 13). Additivity indicates these are independently acting variants.

As shown in Fig. 14, a further variant with improved C1q binding (50% increase) was made by changing A327 in a human IgG1 constant region to glycine. Conversely, in a human IgG2 constant region, changing G327 to alanine reduced C1q binding of the IgG2 antibody.

EXAMPLE 4

Identification of FcR Binding Sites in Human IgG Antibodies

In the present study, the effect of mutating various Fc region residues of an IgG1 antibody with respect to binding Fc γ RI, Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA as well as FcRn was evaluated. Antibody variants with improved as well as diminished FcR binding were identified.

MATERIALS AND METHODS

Construction of IgG1 Variants: Recombinant anti-IgE E27 having the light chain and heavy chain sequences in Figs. 4A and 4B, respectively, was used as the parent antibody in the following experiments. This antibody binds the antigen IgE and has a non-A allotype IgG1 Fc region. By site directed mutagenesis (Kunkel *et al.*, *Proc. Natl. Acad. Sci. USA* 82:488 (1987)), variants of the Fc region in the heavy chain of the above parent antibody were constructed. The heavy and light chain plasmids were co-transfected into an adenovirus transformed human embryonic kidney cell line as previously described (Werther *et al.*, *J. Immunol.* 157:4986 (1996)). The media was changed to serum-free 24 hours after transfection and the secreted antibody was harvested after five days. The antibodies were purified by Protein G SEPHAROSE® (Pharmacia), buffer exchanged and concentrated to 0.5 ml with PBS using a Centricon-30 (Amicon), and stored at 4°C. Concentration was determined by adsorption at 280 nm using extinction coefficients derived by amino acid composition analysis.

High Affinity Fc γ RIA Binding ELISA: Fc γ RIA was expressed as a GST fusion of His6 tagged extracellular domain in 293 cells and purified by Ni-NTA column chromatography.

To purify Fc γ RIA, supernatant from transfected 293 cells was removed after three days. Protease inhibitors were added; 50 μ L Aprotinin (Sigma)/ 50 mL supernatant, and PMSF (1mM). Supernatants were concentrated to 10 mL in a stirred cell (Amicon), and dialyzed overnight at 4°C against 1 liter column buffer (50 mM Tris pH 8.0, 20mM Imidazole, 300 mM NaCl). Additional dialysis was done the following morning against fresh column buffer for 4 hours at 4°C. The solution was loaded on to a 1mL Ni⁺⁺ column (NTA super flow resin, Qiagen) previously equilibrated with 10 mL column buffer. Columns were washed with 10 mL column buffer, and protein was eluted with 2.5 mL elution buffer (50mM Tris pH 8.0, 250mM Imidazole, 300mM NaCl). Protein was concentrated to 0.5 mL and buffer exchanged into PBS.

Concentrations were determined by adsorption at 280 nm using an extinction coefficient derived by amino acid composition analysis.

Purified receptors were coated onto Nunc F96 maxisorb plates (cat no. 439545) at approximately 150 ng per well by adding 100 μ L of receptor at 1.5 μ g/mL in PBS and incubated for 24 hours at 4°C. Prior to assay, plates were washed 3x with 250 μ L of wash buffer (phosphate buffered saline pH 7.4 containing 0.5% TWEEN 20®) and blocked with 250 μ L of assay buffer (50 mM tris buffered saline, 0.05% TWEEN 20®, 0.5% RIA grade bovine albumin (Sigma A7888), and 2mM EDTA pH 7.4).

100 μ L of E27 was added to the first four wells of the Fc γ RIA subunit coated plated at a concentration of 10 μ g/mL. 80 μ L of assay buffer was added to the next four well followed by 20 μ L of the 10 μ g/mL E27 IgG to give a final concentration of 2 μ g/mL. Plates were incubated at 25°C for 2 hours on an orbital shaker.

For detection, plates were washed 5x with wash buffer to remove unbound antibody. IgG binding to GST-Fc γ RIA was detected by adding 100 μ L horse radish peroxidase (HRP) conjugated protein G (BIORAD) at 1:5000. HRP conjugates were incubated for 1.5 hours at 25°C on an orbital shaker. Plates were washed x5 with wash buffer to remove unbound HRP conjugate. Binding was detected by adding 100 μ L of substrate solution (0.4mg/mL o-phenylenedaimine dihydrochloride, Sigma P6912, 6 mM H₂O₂ in PBS) and incubating for 10 minutes at 25°C. Enzymatic reaction was stopped by the addition of 100 μ L of 4.5 N H₂SO₄ and colorimetric product was measured at 490 nm on a 96 well plate densitometer (Molecular Devices).

Binding of E27 variants at IgG concentration of 2 μ g/mL was expressed as a ratio of wild type E27.

Fc γ RIA THP-1 Assay: 100 μ L of E27 was added to the first three wells of a serocluster plate (Costar) at a concentration of 20 μ g/mL in assay buffer (1x PBS, 0.1% BSA, 0.01% NaN₃). 92.5 μ L of assay buffer was added to the next three wells followed by 7.5 μ L of the 20 μ g/mL E27 IgG to give a final concentration of 1.5 μ g/mL. To each well, 100 μ L of THP-1 cells were added at a concentration of 5 million cells/mL in FACS assay buffer. The plate is incubated on ice for 30 minutes

For detection, cells were washed 2x with assay buffer to remove unbound antibody. IgG binding Fc γ RIA was detected by adding 100 μ L FITC conjugated F(ab')₂ fragment of goat anti-human IgG heavy chain specific. (Jackson ImmunoResearch) at 1:200. FITC conjugates were incubated with cells for 30 minutes on ice. Cells were washed x3 with assay buffer to remove unbound FITC conjugate. Cells were stained with P.I. (SIGMA) at 2.5 μ g/mL and analyzed by

flow cytometry.

Binding of E27 variants at IgG concentration of 1.5 $\mu\text{g/mL}$ was expressed as a ratio of wild type E27.

Data from the plate assay (Fc γ RIA ELISA) and cell-based assay (Fc γ RIA THP-1 assay) was averaged to arrive at an Fc γ RIA-binding activity.

Low Affinity Fc γ R Binding ELISAs: Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA binding ELISAs were performed as described in Example 1 above, with detection of the stable hexamer (consisting of three anti-IgE molecules and three IgE molecules).

Binding values: For all Fc γ R, binding values reported in Table 6 are the binding of each E27 variant relative to native E27, taken as (A_{490nm} variant/A_{490nm} native IgG1) at 0.33 or 1 $\mu\text{g/ml}$ for Fc γ RII and Fc γ RIIIA and 2 $\mu\text{g/ml}$ for Fc γ RI. A value greater than 1 denotes binding of the variant was improved compared to native IgG1 while a ratio less than 1 denotes reduced binding compared to native IgG1. Reduced binding to any given receptor was defined as a reduction of $\geq 40\%$ compared to native IgG; better binding was defined as an improvement of $\geq 25\%$ compared to native IgG1. The latter was chosen based on the observation that variants with $\geq 25\%$ improved binding in the ELISA-format assay, such as Glu333Ala, Lys334Ala and Ser298Ala, also showed improved efficacy in the cell-based binding and ADCC assays.

FcRn Binding ELISA: For measuring FcRn binding activity of IgG variants, ELISA plates were coated with 2 $\mu\text{g/ml}$ streptavidin (Zymed, South San Francisco) in 50 mM carbonate buffer, pH 9.6, at 4°C overnight and blocked with PBS-0.5% BSA, pH 7.2 at room temperature for one hour. Biotinylated FcRn (prepared using biotin-X-NHS from Research Organics, Cleveland, OH and used at 1-2 $\mu\text{g/ml}$) in PBS-0.5% BSA, 0.05% polysorbate 20, pH 7.2, was added to the plate and incubated for one hour. Two fold serial dilutions of IgG standard (1.6-100 ng/ml) or variants in PBS-0.5% BSA, 0.05% polysorbate 20, pH 6.0, were added to the plate and incubated for two hours. Bound IgG was detected using peroxidase labeled goat F(ab')₂ anti-human IgG F(ab')₂ in the above pH 6.0 buffer (Jackson ImmunoResearch, West Grove, PA) followed by 3,3',5,5'-tetramethyl benzidine (Kirgaard & Perry Laboratories) as the substrate. Plates were washed between steps with PBS-0.05% TWEEN 20® at either pH 7.2 or 6.0. Absorbance was read at 450 nm on a Vmax plate reader (Molecular Devices, Menlo Park, CA). Titration curves were fit with a four-parameter nonlinear regression curve-fitting program (KaleidaGraph, Synergy software, Reading, PA). Concentrations of IgG variants corresponding to the mid-point absorbance of the titration curve of the standard were calculated and then divided by the concentration of the standard corresponding to the mid-point absorbance of the standard titration curve.

In Vitro ADCC Assay: To prepare chromium 51-labeled target cells, tumor cell lines

were grown in tissue culture plates and harvested using sterile 10 mM EDTA in PBS. SK-BR-3 cells, a 3+ HER2-overexpressing human breast cancer cell line, were used as targets in all assays. The detached cells were washed twice with cell culture medium. Cells (5×10^6) were labeled with 200 μ Ci of chromium51 (New England Nuclear/DuPont) at 37°C for one hour with occasional mixing. Labeled cells were washed three times with cell culture medium, then were resuspended to a concentration of 1×10^5 cells/mL. Cells were used either without opsonization, or were opsonized prior to the assay by incubation with rhuMAb HER2 wildtype (HERCEPTIN®) or seven Fc mutants (G14 D265A; G18 D270A; G17 E269A; G36 S298A; G30 K290A; G31 R292A; and G34 Q295A) at 100 ng/mL and 1.25 ng/mL in PBMC assay or 20 ng/mL and 1 ng/mL in NK assay.

Peripheral blood mononuclear cells (PBMCs) were prepared by collecting blood on heparin from normal healthy donors and dilution with an equal volume of phosphate buffered saline (PBS). The blood was then layered over LYMPHOCYTE SEPARATION MEDIUM® (LSM: Organon Teknika) and centrifuged according to the manufacturer's instructions. Mononuclear cells were collected from the LSM-plasma interface and were washed three times with PBS. Effector cells were suspended in cell culture medium to a final concentration of 1×10^7 cells/mL.

After purification through LSM, natural killer (NK) cells were isolated from PBMCs by negative selection using an NK cell isolation kit and a magnetic column (Miltenyi Biotec) according to the manufacturer's instructions. Isolated NK cells were collected, washed and resuspended in cell culture medium to a concentration of 2×10^6 cells/mL. The identity of the NK cells was confirmed by flow cytometric analysis.

Varying effector:target ratios were prepared by serially diluting the effector (either PBMC or NK) cells two-fold along the rows of a microtiter plate (100 μ L final volume) in cell culture medium. The concentration of effector cells ranged from 1.0×10^7 /mL to 2.0×10^4 /mL for PBMC and from 2.0×10^6 /mL to 3.9×10^3 /mL for NK. After titration of effector cells, 100 μ L of chromium 51-labeled target cells (opsonized or nonopsonized) at 1×10^5 cells/mL were added to each well of the plate. This resulted in an initial effector:target ratio of 100:1 for PBMC and 20:1 for NK cells. All assays were run in duplicate, and each plate contained controls for both spontaneous lysis (no effector cells) and total lysis (target cells plus 100 μ L) 1% sodium dodecyl sulfate, 1 N sodium hydroxide). The plates were incubated at 37°C for 18 hours, after which the cell culture supernatants were harvested using a supernatant collection system (Skatron Instrument, Inc.) and counted in a Minaxi auto-gamma 5000 series gamma counter (Packard) for one minute. Results were then expressed as percent cytotoxicity using the formula: % Cytotoxicity = (sample cpm-spontaneous lysis)/(total lysis-spontaneous lysis) x 100. Four-parameter curve-fitting was then used to evaluate the data (KaleidaGraph 3.0.5).

RESULTS

A variety of antibody variants were generated which had FcR binding activity that differed from the parent antibody. The FcR binding data for the variants generated is shown in Tables 6 and 7 below. An additional variant, T307Q, also displayed improved FcRn binding compared to E27

5 parent antibody.

TABLE 6
Binding of Human IgG1 Variants to Human FcRn and FcγR

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Variant ^a	FcRn ^b	N	FcγRI	N	FcγRIIA	FcγRIIB	FcγRIIIA	NC
mean (sd)	mean (sd)	mean (sd)	mean (sd)	mean (sd)	mean (sd)	mean (sd)	mean (sd)	
Class 1 = Reduced binding to all FcγR								
Glu233Pro	0.54 (0.20)	3	0.12 (0.06)	6	0.08 (0.01)	0.12 (0.01)	0.04 (0.02)	2
Leu234Val								
Leu235Ala								
Gly236deleted								
Pro238Ala	1.49 (0.17)	3	0.60 (0.05)	5	0.38 (0.14)	0.36 (0.15)	0.07 (0.05)	4
Asp265Ala	1.23 (0.14)	4	0.16 (0.05)	9	0.07 (0.01)	0.13 (0.05)	0.09 (0.06)	4
Asn297Ala	0.80 (0.18)	8	0.15 (0.06)	7	0.05 (0.00)	0.10 (0.02)	0.03 (0.01)	3
Ala327Gln	0.97		0.60 (0.12)	9	0.13 (0.03)	0.14 (0.03)	0.06 (0.01)	4
Pro329Ala	0.80		0.48 (0.10)	6	0.08 (0.02)	0.12 (0.08)	0.21 (0.03)	4
Class 2 = Reduced binding to FcγRII and FcγRIIIA								
Asp270Ala	1.05		0.76 (0.12)	6	0.06 (0.02)	0.10 (0.06)	0.14 (0.04)	6
Gln295Ala	0.79		1.00 (0.11)	4	0.62 (0.20)	0.50 (0.24)	0.25 (0.09)	5
Ala327Ser			0.86 (0.03)	4	0.23 (0.06)	0.22 (0.05)	0.06 (0.01)	4
Class 3 = Improved binding to FcγRII and FcγRIIIA								
Thr256Ala	1.91 (0.43)	6	1.01 (0.07)	5	1.41 (0.27)	2.06 (0.66)	1.32 (0.19)	9
Lys290Ala	0.79 (0.14)	3	1.01 (0.06)	11	1.30 (0.21)	1.38 (0.17)	1.31 (0.19)	9
Class 4 = Improved binding to FcγRII and no effect on FcγRIIIA								

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5	Arg255Ala	0.59 (0.19)	4	0.99 (0.12)	7	1.30 (0.20)	1.59 (0.42)	0.99 (0.18)	5
	Glu259Ala	1.18		1.18 (0.13)	4	1.33 (0.22)	1.65 (0.39)	1.12 (0.12)	5
	Ser267Ala	1.08		1.09 (0.08)	10	1.52 (0.22)	1.84 (0.43)	1.05 (0.24)	11
	Glu272Ala	1.34 (0.24)	4	1.05 (0.06)	7	1.23 (0.12)	1.53 (0.22)	0.90 (0.18)	6
10	Asn276Ala	1.15 (0.21)	3	1.05 (0.14)	4	1.29 (0.20)	1.34 (0.40)	0.95 (0.04)	4
	Asp290Ala	0.82		1.04 (0.08)	10	1.34 (0.14)	1.60 (0.31)	1.09 (0.20)	10
	His295Ala	0.85		0.96 (0.07)	4	1.26 (0.12)	1.23 (0.15)	0.97 (0.04)	4
15	Asn296Ala	1.24 (0.04)	2	0.95 (0.18)	16	1.24 (0.23)	1.36 (0.15)	1.05 (0.04)	6
	Thr207Ala	1.81 (0.22)	6	0.99 (0.14)	4	1.07 (0.15)	1.27 (0.24)	1.09 (0.18)	10
20	Leu309Ala	0.63 (0.18)	4	0.93 (0.18)	6	1.13 (0.08)	1.26 (0.12)	1.07 (0.20)	3
	Asn315Ala	0.76 (0.14)	3	0.99 (0.16)	6	1.15 (0.06)	1.30 (0.17)	1.07 (0.21)	5
	Lys326Ala	1.03		1.03 (0.05)	10	1.23 (0.20)	1.41 (0.27)	1.23 (0.23)	7
25	Pro331Ala	0.85		1.01 (0.09)	7	1.29 (0.14)	1.34 (0.35)	1.08 (0.19)	4
	Ser337Ala	1.03		1.17 (0.23)	3	1.22 (0.30)	1.26 (0.06)	0.94 (0.19)	4
30	Ala378Gln	1.32 (0.13)	3	1.06 (0.05)	3	1.40 (0.17)	1.45 (0.17)	1.19 (0.17)	5
	Glu430Ala	0.93 (0.03)	2	1.05 (0.02)	3	1.24 (0.11)	1.29 (0.10)	1.20 (0.19)	5
35	Class 5 = Improved binding to FcγRII and reduced binding to FcγRIIIA								
	His269Ala	1.02 (0.22)	3	1.09 (0.11)	8	1.21 (0.14)	1.44 (0.22)	0.54 (0.12)	13
	Arg301Ala	0.86		1.06 (0.10)	4	1.14 (0.13)	1.29 (0.16)	0.22 (0.08)	7
40	Lys222Ala	0.98		0.94 (0.04)	9	1.17 (0.11)	1.28 (0.21)	0.62 (0.12)	6
	Class 6 = Reduced binding to FcγRII and no effect on FcγRIIIA								

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Arg292Ala

0.81 (0.18) 4 0.95 (0.05) 8 0.27 (0.13) 0.17 (0.07) 0.89 (0.17) 10

Lys414Ala 1.02 1.00 (0.04) 3 0.64 (0.15) 0.58 (0.18) 0.82 (0.27) 3

Class 7 = Reduced binding to FcγRII and improved binding to FcγRIIIA

Ser298Ala 0.80 1.11 (0.03) 9 0.40 (0.15) 0.23 (0.13) 1.24 (0.20) 16

10 Class 8 = No effect on FcγRII and reduced binding to FcγRIIIA

Ser239Ala 1.06 0.81 (0.09) 7 0.73 (0.25) 0.76 (0.36) 0.26 (0.08) 3

Glu269Ala 1.05 0.61 (0.14) 9 0.65 (0.18) 0.75 (0.29) 0.45 (0.13) 5

15 Glu293Ala 0.85 1.11 (0.07) 4 1.08 (0.19) 1.07 (0.20) 0.31 (0.13) 6

Tyr296Phe 0.79 1.03 (0.09) 8 0.97 (0.23) 0.86 (0.17) 0.55 (0.12) 6

20 Val303Ala 1.26 (0.21) 3 0.91 (0.11) 5 0.86 (0.10) 0.65 (0.17) 0.33 (0.09) 8

Ala327Gly 0.96 (0.01) 3 0.92 (0.09) 0.83 (0.10) 0.36 (0.05) 3

25 Lys338Ala 1.14 0.90 (0.05) 3 0.78 (0.09) 0.63 (0.08) 0.15 (0.01) 2

Asp376Ala 1.45 (0.36) 4 1.00 (0.05) 3 0.80 (0.16) 0.68 (0.14) 0.55 (0.10) 5

Class 9 = No effect on FcγRII and improved binding to FcγRIIIA

Glu333Ala

1.03 (0.01) 2 0.98 (0.15) 5 0.92 (0.12) 0.76 (0.11) 1.27 (0.17) 10

Lys334Ala 1.05 (0.03) 2 1.06 (0.06) 11 1.01 (0.15) 0.90 (0.12) 1.29 (0.19) 16

Ala339Thr 1.06 (0.04) 6 1.09 (0.03) 1.20 (0.03) 1.24 (0.09) 2

Class 10 = Effect only FcRn

Ile253Ala

0.10 0.96 (0.05) 4 1.14 (0.02) 1.18 (0.06) 1.08 (0.14) 3

40 Ser254Ala < 0.10 0.96 (0.08) 4 0.97 (0.24) 1.15 (0.38) 0.73 (0.14) 3

Lys299Ala 0.38 (0.12) 5 0.88 (0.15) 15 1.15 (0.26) 1.14 (0.20) 1.06 (0.04) 4

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5	Val305Ala	1.46 (0.48)	6	1.04 (0.19)	10	1.12 (0.12)	1.23 (0.22)	0.94 (0.15)	4
	Gln311Ala	1.62 (0.25)	4	0.93 (0.05)	4	1.11 (0.06)	1.19 (0.13)	0.93 (0.17)	3
	Asp312Ala	1.50 (0.06)	4	1.01 (0.12)	5	1.20 (0.24)	1.19 (0.07)	1.23 (0.14)	3
	Lys317Ala	1.44 (0.18)	4	0.92 (0.17)	6	1.13 (0.05)	1.18 (0.27)	1.10 (0.23)	4
10	Lys336Ala	1.30 (0.09)	4	1.02 (0.04)	3	1.12 (0.10)	1.12 (0.09)	1.23 (0.16)	6
	Gln362Ala	1.25 (0.24)	3	1.00 (0.04)	3	1.03 (0.10)	1.02 (0.02)	1.02 (0.16)	4
15	Glu390Ala	2.19 (0.29)	6	1.04 (0.06)	3	1.18 (0.01)	1.07 (0.05)	0.92 (0.12)	2
	Glu382Ala	1.51 (0.18)	4	1.06 (0.03)	3	0.95 (0.11)	0.84 (0.04)	0.76 (0.17)	3
	Ser415Ala	0.44		1.04 (0.03)	3	0.90 (0.11)	0.88 (0.05)	0.86 (0.16)	2
20	Ser424Ala	1.41 (0.14)	3	0.98 (0.03)	3	1.04 (0.06)	1.02 (0.02)	0.98 (0.02)	2
	His432Ala	0.41 (0.14)	2	0.98 (0.03)	3	0.92 (0.18)	0.79 (0.19)	1.02 (0.15)	3
25	Asn434Ala	3.46 (0.37)	7	1.00 (0.04)	3	0.96 (0.06)	0.97 (0.12)	0.77 (0.13)	6
	His435Ala	< 0.10	4	1.25 (0.09)	3	0.77 (0.05)	0.72 (0.05)	0.79 (0.03)	3
30	Tyr436Ala	< 0.10	2	0.99 (0.02)	2	0.93 (0.05)	0.91 (0.06)	0.91 (0.15)	3
	=====								
	a Residue numbers are according to the Eu numbering system (Kabat et al. (1991)). Variants that had no effect on binding (i.e. did not reduce binding by more than 60% or improve binding by more than 20%) to FcγR or Fcγn were: Lys246, Lys248, Asp249, Met252, Thr260, Lys274, Tyr279, Val292, Glu333, Thr339, Glu394, Tyr300Phe, Glu319, Lys320, Ser324, Ala320Gln, Thr335, Lys340, Gln342, Arg344, Glu345, Gln347, Arg355, Glu356, Met358, Thr359, Lys360, Asn361, Tyr373, Ser375, Ser383, Asn384, Gln386, Glu388, Asn389, Asn390, Tyr391Phe, Lys392, Leu398, Ser400, Asp401, Asp413, Arg416, Gln418, Asn422, Val422, Thr437, Gln438, Lys439, Ser440, Ser442, Ser444, Lys447.								
35	b Values are the ratio of binding of the variant to that of native IgG1 at 0.33 or 1 ug/ml. A value greater than 1 denotes binding of the variant was improved compared to native IgG1 while a ratio less than 1 denotes reduced binding compared to native IgG1. Reduced binding to any given receptor was defined as a reduction of 24% compared to native IgG; better binding was defined as an improvement of 25% compared to native IgG1.								
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c Number of independent assays for FcγRIIA, FcγRIIB and FcγRIIA. At least two separately expressed and purified lots of each variant were assayed.

Aside from alanine variants, various non-alanine substitution variants were made, and the FcR binding activity of those variants is summarized in the following table.

TABLE 7
NON-ALANINE VARIANTS

IG2	Res#EU (Kabat)	FCRn	mean	sd	n	FcγRI	mean	sd	n	FcγRIIA ³	mean	sd	n	FcγRIIB	mean	sd	n	FcγRIIA	mean	sd	n
222	D249(262)E						0.97				0.99				0.94			n=1			
176	T256(269)G						1.10	(0.03)			1.06	(0.07)			0.96	(0.27)		n=2			
254	T256(269)N						1.03				0.89				1.13			n=1			
157	D265(278)N						0.02	(0.01)			0.03	(0.01)			0.02	(0.01)		n=3			
158	D265(278)E						0.11	(0.04)			0.03	(0.01)			0.02	(0.01)		n=3			
189	S267(290)G						R131 1.18 (0.10)				0.95	(0.16)			0.08	(0.02)		n=4			
							H131 0.59 (0.09)											n=3			
84	H268(281)N						1.33				1.41				0.56			n=1			
85	H268(281)S						1.35				1.38				0.81			n=1			
87	H268(281)Y						1.19				1.29				0.76			n=1			
168	E269(292)D						0.89	(0.10)			0.73	(0.07)			1.13	(0.21)		n=2			
169	E269(292)Q						0.08	(0.01)			0.16	(0.00)			0.29	(0.03)		n=2			
92	D270(293)N						0.03	(0.02)			0.05	(0.05)			0.04	(0.03)		n=5			
93	D270(293)E						0.08	(0.01)			0.06	(0.01)			0.90	(0.17)		n=3			
223	E272(295)Q						1.93				1.81				0.82			n=1			
224	E272(295)N						0.43				0.23				0.50			n=1			

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167	K274(287)Q	0.86	0.94	0.62	n=1
165	N276(289)K	0.81	0.77	0.61	n=1
5	233 N276(289)Q	1.09	0.79	0.91	n=1
79	D290(295)N	1.07 (0.18)	1.22 (0.19)	1.16 (0.21)	n=6
149	D290(295)S	1.07 (0.06)	1.04 (0.08)	1.09 (0.06)	n=2
226	E293(300)Q	1.12	1.24	1.19	n=1
227	E293(300)S	1.03	1.07	0.85	n=1
228	E293(300)N	1.18	1.28	0.94	n=1
229	E293(300)D	1.14	1.23	0.95	n=1
23	N296(303)Q	1.52	1.13	0.96	n=1
237	N296(303)S	1.72	1.38	1.32	n=1
238	N296(303)D	1.41	1.23	0.98	n=1
73	K290(307)Q	1.17	1.26	1.40	n=1
75	K290(307)S	1.27	1.34	1.26	n=1
77	K290(307)E	1.10	1.20	1.30	n=1
78	K290(307)R	1.05	1.15	1.08	n=1
177	K290(307)G	1.07	1.21	1.23	n=1
80	E292(309)K	0.71 (0.17)	0.75 (0.10)	1.15 (0.18)	n=3
81	E292(309)H	0.21 (0.09)	0.12 (0.01)	0.92 (0.09)	n=2
82	E292(309)Q	0.47 (0.12)	0.25 (0.06)	0.45 (0.09)	n=3
83	R292(309)N	0.54 (0.16)	0.29 (0.07)	0.88 (0.02)	n=3
144	E293(310)Q	0.85 (0.03)	0.77 (0.12)	0.99 (0.04)	n=2
145	E293(310)D	0.90 (0.02)	0.88 (0.07)	0.37 (0.07)	n=3

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147	E293(31C)K	1.13 (0.04)	1.31 (0.17)	0.72 (0.08)	n=4
173	E294(311)Q	1.01	0.95	0.84	n=1
174	E294(311)D	0.37	0.26	0.14	n=1
185	Y296(213)H	0.90	0.81	0.22	n=1
186	Y296(313)W	0.96	0.93	1.38	n=1
70	S298(317)G	0.87 (0.17)	0.63 (0.33)	0.46 (0.09)	n=4
71	S298(317)T	0.29 (0.19)	0.27 (0.19)	0.73 (0.21)	n=6
72	S298(317)N	0.05 (0.03)	0.08 (0.08)	0.06 (0.03)	n=5
218	S298(317)V	0.11 (0.06)	0.17 (0.01)	0.33 (0.19)	n=3
219	S298(317)L	1.14 (0.12)	1.42 (0.31)	0.34 (0.04)	n=3
150	V303(322)L	0.89 (0.05)	0.73 (0.10)	0.76 (0.09)	n=4
151	V303(322)T	0.64 (0.11)	0.34 (0.05)	0.20 (0.05)	n=4
217	E319(337)K	1.03	1.08	0.72	n=1
172	K320(339)R	0.71	0.66	0.68	n=1
202	K320(339)M	1.34	1.40	1.27	n=1
204	K320(339)Q	1.23	1.12	1.17	n=1
205	K320(339)E	1.29	1.34	1.12	n=1
235	K320(329)F	1.24	0.95	0.96	n=1
155	K322(341)R	0.87 (0.07)	0.87 (0.21)	0.92 (0.15)	n=3
156	K322(341)Q	0.87 (0.02)	0.92 (0.22)	0.79 (0.19)	n=3
206	K322(341)E	1.38	1.34	0.81	n=1
207	K322(341)N	0.57	0.36	0.04	n=1

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213	S324(343)N	1.15	1.09	0.97	n=1
214	S324(343)Q	0.82	0.83	0.78	n=1
215	S324(343)K	0.66	0.37	0.77	n=1
216	S324(343)E	0.82	0.73	0.81	n=1
208	K326(345)S	1.44	1.62	1.37	n=1
209	K326(345)N	1.04	1.00	1.27	n=1
210	K326(345)Q	1.36	1.41	1.15	n=1
211	K326(345)D	1.68	2.01	1.36	n=1
212	K326(345)E	1.34 (0.27)	1.47 (0.32)	1.26 (0.04)	n=1
131	A327(345)S	0.23 (0.06)	0.22 (0.05)	0.06 (0.01)	n=4
159	A327(345)G	0.92 (0.09)	0.83 (0.10)	0.36 (0.05)	n=2
196	A330(349)D	0.18	0.08	0.07	n=1
197	A330(349)K	1.28	1.25	1.28	n=1
198	P331(350)S	0.91 (0.08)	0.78 (0.07)	0.58 (0.19)	n=4
199	P331(350)N	0.86	0.65	0.23	n=1
200	P331(350)E	1.06	0.91	0.42	n=1
203	P331(350)K	0.94	0.71	0.33	n=1
141	E333(352)Q	0.70 (0.05)	0.64 (0.09)	1.05 (0.09)	n=3
142	E333(352)N	0.59 (0.04)	0.52 (0.07)	0.56 (0.10)	n=4
143	E333(352)S			0.94	n=1
152	E333(352)K			0.85 (0.14)	n=3
153	E333(352)R	0.75 (0.04)	0.66 (0.03)	0.84 (0.05)	n=2
154	E333(352)D			1.26 (0.04)	n=3

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178	E333 (352)G	0.87	0.76	1.05	n=1
179	K334 (353)G	0.76 (0.08)	0.60 (0.13)	0.88 (0.22)	n=5
135	K334 (353)R	1.15 (0.09)	1.33 (0.18)	0.68 (0.07)	n=5
136	K334 (353)Q	1.08 (0.11)	1.10 (0.21)	1.23 (0.09)	n=7
137	K334 (353)N	1.16 (0.11)	1.29 (0.30)	1.11 (0.12)	n=8
138	K334 (353)S	1.01 (0.11)	1.03 (0.05)	1.19 (0.08)	n=3
139	K334 (353)E	0.74 (0.15)	0.72 (0.12)	1.30 (0.09)	n=6
140	K334 (353)D	0.51 (0.09)	0.40 (0.03)	1.13 (0.09)	n=4
190	K334 (353)M	1.18	0.93 (0.15)	0.49 (0.04)	n=2
191	K334 (353)Y	1.15	1.05	1.31	n=1
192	K334 (353)W	1.16	0.91	1.07	n=1
193	K334 (353)H	1.11	1.07	1.26	n=1
220	K334 (353)V	1.13 (0.11)	1.09 (0.15)	1.34 (0.18)	n=3
221	K334 (353)L	1.05	1.09	1.38	n=1
171	T335 (354)Q	0.86	0.79	0.84	n=1
194	T335 (354)E	1.24	1.30	1.19	n=1
195	T335 (354)K	1.19	1.14	1.30	n=1
273	A339 (359)T	1.23	1.23	1.42	n=1
	S267 (280)T	0.42 (0.10)	0.45 (0.01)	0.05 (0.05)	n=3
	R201 (320)M	1.29 (0.17)	0.48 (0.21)	n=4	
	K338 (358)M	0.99 (0.13)	0.49 (0.04)	n=2	

45 a Values are the ratio of binding of the variant to that of native IgG1 at 0.33 or 1 µg/ml.

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A value greater than 1 denotes binding of the variant was improved compared to native IgG1 while a ratio less than 1 denotes reduced binding compared to native IgG1.

- 5 b Number of independent assays for FcγRIIA, FcγRIIB and FcγRIIIA. At least two separately expressed and purified lots of each variant were assayed.

The following table summarizes the FcR binding activity of various combination variants.

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TABLE 8
COMBINATION VARIANTS

	IG2	Res#EU (Kabat)	FcRn ^a		FcγRI		FcγRIIA [†]		FcγRIIB		FcγRIIA	
			mean	sd	n	mean	sd	n	mean	sd	mean	sd
5	96	S267(280)A H268(281)A	1.41	(0.00)		1.56	(0.16)		0.96	(0.12)		n=2
10	134	E333(352)A K334(353)A	0.72	(0.08)		0.63	(0.13)		1.30	(0.12)		n=5
15	1059	T256(269)A S298(317)A	0.44	(0.03)		0.22	(0.04)		1.41	(0.06)		n=2
	1051	T256(269)A D280(295)A S298(317)A T307(326)A	0.47	(0.01)		0.30	(0.02)		1.21	(0.26)		n=2
20	106	T256(269)A D280(295)A R292(309)A S298(317)A T307(326)A	0.11			0.08			0.90			n=1
25	107	S298(317)A E333(352)A	0.34	(0.05)		0.16	(0.08)		1.53	(0.24)		n=5
30	109	S298(317)A K334(353)A	0.41	(0.07)		0.19	(0.09)		1.62	(0.34)		n=6
35	110	S298(317)A E333(352)A K334(353)A	0.34	(0.15)		0.15	(0.06)		1.51	(0.31)		n=10
	246	S267(280)A E258(271)A	1.62	(0.15)		2.01	(0.45)		1.04	(0.12)		n=2
40	247	S267(280)A R255(268)A	1.60	(0.18)		1.72	(0.13)		0.88	(0.07)		n=3
	248	S267(280)A D280(295)A	1.54	(0.08)		1.96	(0.37)		1.13	(0.07)		n=2
45												

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250	S267(280)A E272(285)A	1.51 (0.13)	1.82 (0.32)	0.95 (0.05)	n=3
251	S267(280)A E293(310)A	1.67 (0.11)	1.85 (0.10)	0.92 (0.09)	n=3
264	S267(290)A E258(271)A D280(295)A R255(268)A	1.48 (0.12)	2.03 (0.30)	0.89 (0.04)	n=2
269	E280(405)A N434(465)A	1.02 (0.07)	1.05 (0.11)	1.02	n=2
270	E390(405)A N434(465)A T307(326)A	0.99 (0.06)	0.99 (0.11)	0.96	n=2
271	E380(405)A L309(328)A	0.98	1.04	0.92	n=1
272	N424(465)A K288(305)A	0.94 (0.11)	0.96 (0.17)	0.89	n=2

a. Values are the ratio of binding of the variant to that of native IgG1 at pH 6.0.

b. Values are the ratio of binding of the variant to that of native IgG1 at 0.33 or 1 µg/ml. A value greater than 1 denotes binding of the variant was improved compared to native IgG1 while a ratio less than 1 denotes reduced binding compared to native IgG1.

DISCUSSION

This study includes a complete mapping of human IgG1 for human Fc γ R1, Fc γ R1IA, Fc γ R1IB, Fc γ R1IIIA, and FcRn. An alanine-scan of all amino acids in human IgG1 Fc (CH2 and CH3 domains) exposed to solvent, based on the crystal structure of human Fc (Deisenhofer, *Biochemistry* 20:2361-2370 (1981)), was performed. Each exposed amino acid in CH2 and CH3 was individually changed to alanine and the variant IgG assayed against all five human receptors; all variants were evaluated using humanized anti-IgE E27 IgG1 as the parent polypeptide. Fc γ R1 and FcRn are high affinity receptors and monomeric IgG could be evaluated in the assays for these two receptors. Fc γ R1IA, Fc γ R1IB and Fc γ R1IIIA are low affinity receptors and required use of an immune complex. Hence, an ELISA-type assay was used for Fc γ R1IA, Fc γ R1IB, and Fc γ R1IIIA, in which pre-formed hexamers, consisting of three anti-IgE E27 and three IgE molecules were bound to the Fc γ R and either anti-human IgG Fc-HRP or protein G-HRP used as detection reagent. In order to increase binding, these hexamers could be linked into multimers by addition of human VEGF (using anti-VEGF IgE). The hexamers bound to the low affinity Fc γ R significantly better than the IgG monomers; the multimers bound better than the hexamers (Figs. 15A and 15B). The hexameric complexes were used since these provided sufficient binding and required less IgG. Complexes formed using other antibody:antigen combinations are also possible reagents, as long as the antigen contains at least two identical binding sites per molecule for the antibody. As an example, VEGF contains two binding sites per VEGF dimer for anti-VEGF A.4.6.1 (Kim *et al.*, *Growth Factors* 7:53 (1992) and Kim *et al.* *Nature* 362:841 (1993)). VEGF:anti-VEGF multimers also bound to the low affinity Fc γ R1IA and Fc γ R1IIIA (Figs. 16A and 16B).

Classes of Fc Region Variants

Once the complete alanine-scan was performed, several classes of alanine variants were found. Some variants exhibited reduced binding to all Fc γ R (G14 D265A; Fig. 17), while other variants showed reduced binding only to one Fc γ R (G16 H268A; Fig. 17), improved binding only to one Fc γ R (G15 S267A; G54 E333A; G55 K334A; Fig. 17), or simultaneous reduction to one Fc γ R with improvement to another (G36 S298A; Fig. 17).

The IgG1 variants can be separated into distinct classes based on their effects on binding to the various receptors. Class 1 consists of variants which showed reduced binding to all Fc γ R (Table 6) and are clustered near the region of the CH2 domain where the hinge joins CH2 (Fig. 26A). In the Glu233Pro/Leu234Val/Leu235Ala/Gly236 deleted variant, part of the so-called lower hinge region (residues 233-239) of human IgG1 was exchanged with that of human IgG2. The reduction in binding to all Fc γ R is in agreement with previous studies; this variant also showed impaired binding to FcRn. Two other residues in the lower hinge region were individually changed to Ala; Pro238Ala (Class 1) had a more pronounced effect than Ser239Ala (Class 8). If the Pro238Ala effect is due to a conformation change, this change was beneficial

for binding to FcRn. In contrast, Pro329Ala showed a relatively modest reduction in binding to FcRn compared to the significant reduction in binding to the FcγR. In the IgG1 Fc:FcγRIIIA crystal structure (Sondermann *et al. Nature* 406:267-273 (2000)), Pro329 intimately interacts with two Trp sidechains of the receptor and the loss of these interactions by Pro329Ala may account for the severe reduction in binding. Pro329 is also involved in binding of human IgG1 to human C1q (Example 2 herein).

Removal of the conserved Asn-linked glycosylation site in the CH2 domain, Asn297Ala, abolished binding, in agreement with earlier studies (Lund *et al. J. Immunol.* 157: 4963-4969 (1996); and Lund *et al. FASEB J.* 9: 115-119 (1995)). Another residue which interacts with carbohydrate, Asp265, has also been previously found to be important in human IgG3 binding to human FcγRI (Lund *et al. J. Immunol.* 157: 4963-4969 (1996); and Lund *et al. FASEB J.* 9: 115-119 (1995)). In human IgG1, changing Asp265 to Ala, Asn or Glu nullified binding (Tables 6 and 7), suggesting that both the charge and size are important. The results of the Ala327Gln (Class 1) and Ala327Ser (Class 2) variants imply that the region around Ala327 involved in binding to the FcγR may require a close fit between receptor and IgG1, as enlarging this sidechain diminished binding. This position is an Asp in mouse IgG2a and IgG2b and therefore changing the human IgG1 Ala to a larger sidechain is unlikely to have affected the conformation. The Ala327Gly (Class 8) variant reduced binding only to FcγRIIIA suggesting that this receptor requires the presence of a small amino acid sidechain at this position whereas the other receptors do not.

Class 1 variants (in addition to the hinge residues, which were not investigated in this study) comprise the entire binding site on IgG1 for FcγRI. Residues in the F(ab) portions of the IgG1 do not contribute to FcγRI binding as evidenced by both a CD4-immunoadhesin (Capon *et al. Nature* 337:525-531 (1989)) and an Fc fragment binding to FcγRI as effectively as did intact IgG1. Notably, no variants were found which reduced binding only to FcγRI.

Class 2 consists of three variants with reduced binding to FcγRII and FcγRIII but not FcγRI. Like the residues in Class 1, Asp270 and Gln295 are located near the hinge (Fig. 26A). In crystal structures of human IgG1 Fc (Deisenhofer J., *Biochemistry* 20: 2361-2370 (1981); Guddat *et al., PNAS(USA)* 90: 4271-4275 (1993)), Gln295 is completely solvent exposed whereas Asp270, though exposed, forms hydrogen bonds from its sidechain Oδ atom to the backbone nitrogens of Lys326 and Ala327 and to the sidechain Nδ of Asn325. Disruption of these interactions by Asp270Ala could cause a local conformational perturbation that effected the severe reduction in binding to FcγRII and FcγRIIIA. However, Asp270Ala did not affect binding to FcγRI or FcRn. Furthermore, Asp270Asn, which could maintain the aforementioned hydrogen bonds, also abolished binding to FcγRII and FcγRIIIA, and Asp270Glu bound to FcγRIIIA as effectively as did native IgG1 (Table 7). Taken together, these data suggest that the sidechain charge of Asp270 is important for interaction with FcγRII and FcγRIIIA.

Class 3 consists of two variants with improved binding to FcγRIIA, FcγRIIB, and FcγRIIIA. Thr256 and Lys290 are located near one another in the CH2 domain (Fig. 26). Thr256Ala also exhibited improved binding to FcRn.

Class 4 variants were characterized by improved binding only to FcγRIIA and FcγRIIB.

5 Those that improved binding to FcγRII the most – Arg255Ala, Glu258Ala, Ser267Ala, Glu272Ala, and Asp280Ala – are distant from one another in the CH2 domain (Fig. 26). Of these, only Ser267 was cited as an interacting residue in the Fc:FcγRIIA crystal structure (Sondermann *et al. Nature* 406:267-273 (2000)). Ser267Ala improved binding only to FcγRII, Ser267Gly abolished binding only to FcγRIIIA and Ser267Thr reduced binding to FcγRII and
10 FcγRIIIA (Tables 6 and 7). Asp280Asn (Table 7), like Asp280Ala (Table 6), improved binding only to FcγRII. Three of the Class 4 residues also exhibited improved binding to FcRn (Glu272Ala, Thr307Ala, Ala378Gln) while Arg255Ala exhibited reduced binding. Ala378 interacts with CH2 domain loop AB, which contains residues that interact directly with FcRn, and hence may influence binding to FcRn indirectly.

15 Class 5 variants exhibited improved binding to FcγRIIA and FcγRIIB but, in contrast to Class 4, also showed reduced binding to FcγRIIIA. Of these, Lys322 has also been implicated in human C1q binding (Example 2 above). The aliphatic portion of the Arg301 sidechain is buried and interacts with the Tyr296 sidechain, at least in some crystal structures, while the Arg301 guanidinium group may interact with the Asn297-linked carbohydrate (Deisenhofer, J.
20 *Biochemistry* 20: 2361-2370 (1981); Guddat *et al. Proc. Natl. Acad. Sci. USA* 90: 4271-4275 (1993); and Harris *et al. J. Mol. Biol.* 275: 861-872 (1998)). The Arg301Ala variant effected a modest improvement in binding to FcγRIIB and a pronounced reduction in binding to FcγRIIIA (Table 6); the Arg301Met variant, which may maintain the aliphatic interaction of the Arg301 sidechain, showed improved binding to FcγRIIA and FcγRIIB and a less pronounced reduction
25 of binding to FcγRIIIA compared to Arg301Ala (Table 7).

Class 6 residues show diminished binding to FcγRII only. Arg292Ala is located in the CH2 domain distant from the hinge. Lys414 is at the "bottom" of the IgG1, spatially removed from all other residues having an effect on FcγRII binding, suggesting that it may play only a minor role in binding (discussed below).

30 Class 7 is comprised of Ser298Ala which reduced binding to FcγRII, but improved binding to FcγRIIIA. Situated among the Class 1 residues near the hinge (Fig. 26), Ser298 is also part of the Asn-linked glycosylation sequence Asn297-Ser298-Thr299. Ser298Thr followed the pattern of Ser298Ala, whereas Ser298Asn abolished binding to FcγRIIIA as well as FcγRII (Table 7).

Reduced binding only to FcγRIIIA characterizes Class 8 and includes five residues in the CH2 domain and two in the CH3 domain (Ala327Gln is in Class 1). Ser239 has been previously identified as playing a minor role in murine IgG2b binding to murine FcγRII (Lund *et al. Mol. Immunol.* 29: 53-59 (1992)) and in the IgG1 Fc:FcγRIIIA crystal structure (Sondermann *et al. Nature* 406:267-273 (2000)), the Ser239 in one of the two heavy chains forms a hydrogen-bond to the Lys117 sidechain of FcγRIIIA. In contrast, Glu293Ala (Table 6) and Glu293Asp (Table 7) reduced binding as much as did Ser239Ala even though Glu293 is not located near the Fc:FcγRIIIA interface in the crystal structure. In some crystal structures (Deisenhofer, J. *Biochemistry* 20: 2361-2370 (1981); Guddat *et al. Proc. Natl. Acad. Sci. USA* 90: 4271-4275 (1993); and Harris *et al. J. Mol. Biol.* 275: 861-872 (1998)), the Tyr296 sidechain interacts intimately with the aliphatic portion of Arg301 (Class 5); altering either of these reduced binding to FcγRIIIA. Note, however, that Tyr296 was changed to Phe (not Ala) and the 50% reduction in binding to FcγRIIIA is believed to be due to removal of the sidechain hydroxyl group.

At position Lys338, altering the sidechain to Ala or Met effected reduction in binding to FcγRIIIA, suggesting that both the sidechain charge and aliphatic portions are required. The Lys338 sidechain forms part of the interface between the CH2 and CH3 domains and participates in a salt-bridge with Glu430 in several crystal structures (Deisenhofer, J. *Biochemistry* 20: 2361-2370 (1981); Harris *et al. J. Mol. Biol.* 275: 861-872 (1998); Harris *et al. Biochemistry* 36: 1581-1597 (1997)) (Fig. 26B). While it is possible that altering Lys338 may disrupt the CH2:CH3 interface and thereby influence binding, Lys338Ala (Table 6) and Lys338Met (Table 7) did not disrupt binding to FcγRI, FcγRII or FcRn. Since it is known that binding of IgG1 to FcRn involves residues in both CH2 and CH3 (Raghavan and Bjorkman *Annu. Rev. Cell Dev. Biol.* 12: 181-220 (1996); Ward and Ghetie *Ther. Immunol.* 2: 77-94 (1995); and Burmeister *et al. Nature* 372: 379-383 (1994)), this suggests that any conformational effect of Lys338Ala must be local and minimal. Note also that while Lys338Ala and Lys338Met reduced binding to FcγRIIIA, Glu430Ala (Class 4) improved binding, suggesting that the Lys338:Glu430 salt-bridge is not essential in maintaining binding. Another CH3 residue affecting FcγRIIIA is Asp376 that interacts with the CH2 domain.

Class 9 is characterized by improved binding only to FcγRIIIA and includes Glu333Ala, Lys334Ala and Ala339Thr. A previous study found that Ala339Thr improved binding to FcγRI (Chappel *et al. J. Biol. Chem.* 268: 25124-25131 (1993)); in this study the Ala339Thr variant bound better than native IgG1 to FcγRIIIA but not FcγRI (Table 6). Several non-Ala variants were tested at Glu333 and Lys334. Glu333Asp also improved binding to FcγRIIIA while Glu333Asn reduced binding to FcγRII as well as FcγRIIIA (Table 7). At position 334, changing

Lys to Gln, Glu or Val maintained the improved binding to FcγRIIIA (Table 7). Surprisingly, the Lys334Arg variant reversed the receptor preference, *i.e.* this variant bound better to FcγRIIB, not FcγRIIIA as for the Lys334Ala variant. Taken together these data suggest that FcγRIIIA interacts with Lys334 even though this residue is not among the IgG1 residues found to interact with FcγRIIIA in the co-crystal structure (Sondermann *et al.* *Nature* 406:267-273 (2000)).

Class 10 residues influenced binding only to FcRn. Note that residues in other classes may also have affected binding to FcRn, but were classified according to their effect on FcγR. Positions which effectively abrogated binding to FcRn when changed to alanine include Ile253, Ser254, His435, and Tyr436. Other positions showed a less pronounced reduction in binding: Glu233-Gly236 (Class 1), Arg255 (Class 4), Lys288, Ser415, and His433. Several amino acid positions exhibited an improvement in FcRn binding when changed to alanine; notable among these are Pro238 (Class 1), Thr256 (Class 3), Thr307 (Class 4), Gln311, Asp312, Glu380, Glu382 and Asn434. The pattern of binding was the same when a second assay format was used, *e.g.* with IgE-coated plates rather than FcRn-coated plates.

The set of IgG1 residues involved in binding to all human FcγR are represented by Class 1 (Table 6). Indeed, this set comprises the entire binding site on IgG1 for FcγRI. Class 1 residues are located in the CH2 domain proximal to the hinge and fall into two categories: (1) positions that may interact directly with all FcγR include Leu234-Pro238, Ala327, and Pro329 (and possibly Asp265); (2) positions that influence carbohydrate nature or position include Asp265 and Asn297.

Previous studies mapping the binding residues in mouse or human IgG have concentrated primarily on the lower hinge region, *i.e.* residues Leu234-Ser239, revealing Leu234 and Leu235 as the two most important for FcγRI (Duncan *et al.* *Nature* 332, 563-564 (1988); Canfield and Morrison *J. Exp. Med.* 173:1483-1491 (1991)) and Leu234 and Gly237 as the two most important for FcγRII (Lund *et al.* *Mol. Immunol.* 29: 53-59 (1992); Sondermann *et al.* *Nature* 406: 267-273 (2000)). Of the two residues in the lower hinge investigated in this study, Pro238Ala affected binding to all FcγR while Ser239Ala affected binding only to FcγRIIIA.

In the co-crystal structure of IgG1 Fc:FcγRIIIA (Sondermann *et al.* *Nature* 406: 267-273 (2000)), Pro329 interacts with two Trp sidechains from the receptor and a similar interaction may occur with the other FcγR. However, removal of the Pro sidechain, as in Pro329Ala, might cause a localized conformational change which perturbs adjacent binding residues, supported by the above report that Pro329Ala also effects C1q binding (Example 2). Ala327Gln could be causing steric hindrance to binding due to introduction of a large sidechain at this position, though altering Ala327 to Ser did not affect binding to FcγRI. Inspection of the IgG1 Fc:FcγRIIIA crystal structure shows that Ala327 is near the IgG1:FcγRIIIA interface and forms a van der Waals' interaction with the Trp87 sidechain; however, it is not obvious why introduction of a

larger sidechain such as Ser or Gln should so severely reduce binding. For Asn297 and Asp265, earlier studies evaluated the requirement for carbohydrate attached at Asn297 as well as the influence of Asp265 on the nature of the carbohydrate (Lifely *et al. Glycobiology* 5 :813-822 (1995) ; Lund *et al. J. Immunol.* 157: 4963-4969 (1996); and Lund *et al. FASEB J.* 9: 115-119 (1995)); these will be discussed below.

For Fc γ RI the IgG segment Gly316-Ala339 has also been previously implicated based on sequence comparison and binding of IgG subclasses from different species (Woof *et al. Mol. Immunol.* 23: 319-330 (1986); Burton *et al. Mol. Immunol.* 25: 1175-1181(1988)) and mutagenesis (Canfield and Morrison *J. Exp. Med.* 173: 1483-1491 (1991); Chappel *et al. J. Biol. Chem.* 268: 25124-25131 (1993)). Within the segment Gly316-Ala339, however, only Ala327Gln and Pro329Ala affected binding to Fc γ RI (Class 1). All other exposed residues in the 316-339 segment had no effect. In contrast to a previous study in which changing residue 331 from Pro to Ser in human IgG3 reduced binding by 10-fold (Canfield and Morrison *J. Exp. Med.* 173: 1483-1491 (1991)), in human IgG1 the Pro331Ala (Table 6) and Pro331Ser (Table 7) variants had no effect. Another previous report showed that an Ala339Thr substitution could improve binding to Fc γ RI by 3-fold (Chappel *et al. J. Biol. Chem.* 268: 25124-25131 (1993)); in this study the Ala339Thr variant was only equivalent in binding to native IgG1 (Class 3).

It has been noted that the presence of the γ -chain may augment the binding affinity of the Fc γ RI α -chain (Miller *et al. J. Exp. Med.* 183: 2227-2233 (1996)). Since it is conceivable that some residues in the human IgG1 might interact directly with the γ -chain, binding of the IgG1 variants to Fc γ RI on THP-1 cells was tested as well to the Fc γ RI α -chain coated on a plate. The results of the two assay formats were the same for the entire panel of variants, suggesting that the γ -chain augments binding by the α -chain through a mechanism other than direct interaction with the IgG1.

Since Fc γ RI binds monomeric IgG1 about 100-fold more strongly than do Fc γ RII and Fc γ RIII, one might expect that Fc γ RI would utilize either different or additional IgG1 residues to effect the tighter binding. However, the set of IgG1 residues that control binding to Fc γ RI are a subset of those effecting binding to Fc γ RII and Fc γ RIII (Class 1). This suggests that the comparatively strong binding of IgG1 to Fc γ RI results from either (1) utilization of only two Ig-like domains of Fc γ RI (analogous to the two Ig-like domains of Fc γ RII and Fc γ RIII) but with interaction of different amino acids on Fc γ RI than are used by Fc γ RII and Fc γ RIII, (2) utilization of the same amino acids on all three receptors but with additional direct interaction of amino acids in the third Fc γ RI domain, or (3) the third domain of Fc γ RI effects a conformational change in the other two Ig-like domains which results in more efficacious interaction of these domains with the common set of binding residues on IgG1. In both human and murine Fc γ RI, removal of the third domain reduces affinity for monomeric IgG and alters specificity for IgG subclasses

(Hulett *et al. J. Immunol.* 147: 1863-1868 (1991); Porges *et al. J. Clin. Invest.* 90: 2101-2109 (1992)). This would support, but does not discriminate between, possibilities 2 and 3.

In contrast to Fc γ RI, Fc γ RII requires the presence of two identical IgG heavy chains (Haagen *et al. J. Immunol.* 154: 1852-1860 (1995)), suggesting that residues from both heavy chains may form the Fc γ RII binding site in IgG. The set of IgG1 residues, in addition to the common Class 1 residues, which affect binding to Fc γ RII are: (largest effect) Arg255, Thr256, Glu258, Ser267, Asp270, Glu272, Asp280, Arg292, Ser298 and (less effect) His268, Asn276, His285, Asn286, Lys290, Gln295, Arg301, Thr307, Leu309, Asn315, Lys322, Lys326, Pro331, Ser337, Ala339, Ala378, Lys414.

A previous study elucidated the residues in murine IgG2b involved in binding to murine Fc γ RII (Lund *et al. Mol. Immunol.* 29: 53-59 (1992)). Of the residues investigated in that study, only Asn297Ala and Glu318Ala showed a complete abrogation of binding. Several other murine IgG2b residues exhibited more modest reduction in binding to Fc γ RII: Ser239Ala, Lys248Ala, Ser267Ala, Lys322Ala, Glu333Ala, Thr335Ala, Ser337Ala, and Lys340Ala (Lund *et al. Mol. Immunol.* 29: 53-59 (1992)). Several of these residues also exhibited modest reduction in binding in the human system (*e.g.* Ser239Ala, Thr335Ala) or modest improvement in binding (*e.g.* Lys340Ala) but fell outside of the cutoff used in this study. Noteworthy differences between the two systems are: Asp270Ala affecting only the human system, Glu318Ala affecting only the murine system, and Lys322Ala, Ser267Ala, and Ser337Ala exhibiting improved binding in the human system but slightly reduced binding in the murine.

In contrast to Fc γ RI, several variants exhibited improved binding to Fc γ RIIA and Fc γ RIIB (Classes 3, 4 and 5). Of special interest are Class 4 containing residues which, when changed to Ala, improved binding only to Fc γ RII and Class 5 containing residues which, when changed to Ala, simultaneously improved binding to Fc γ RII and reduced binding to Fc γ RIIA. These can be used to make IgG1 with improved specificity for Fc γ RII over Fc γ RIIA.

Recently the crystal structures of human Fc γ RIIA (Maxwell *et al. Nature Struct. Biol.* 6: 437-442 (1999)) and Fc γ RIIB (Sondermann *et al. EMBO J.* 18: 1095-1103 (1999)) have been solved. In the Fc γ RIIA report, it was suggested that in addition to the lower hinge (Leu234-Gly237), residues in IgG CH2 domain loops FG (residues 326-330) and BC (residues 265-271) might play a role in binding, though it was noted that these had yet to be demonstrated by mutagenesis. Of the four exposed residues in loop FG, Ala330Gln had no effect, Ala327Gln, Ala327Ser and Pro329Ala reduced binding, while Lys326Ala improved binding. Of the five exposed residues in loop BC, two reduced binding when altered to Ala (Asp265Ala and Asp270Ala) and two improved binding (Ser267Ala and His268Ala). Several of the residues found to influence binding to Fc γ RII lie outside of the residues at the Fc:Fc γ RIIA interface in the co-crystal structure (Sondermann *et al. EMBO J.* 18: 1095-1103 (1999)). One of these, Asp280,

is not only distant from the Fc:FcγRIIIA interface but is distant from other FcγRII-influencing residues (Fig. 26A). However, both Asp280Ala and Asp280Asn improved binding to FcγRII, suggesting that this residue does indeed interact with FcγRII.

In addition to the Class 1 residues, positions which reduced binding to FcγRIIIA by 40% or more (when changed to Ala) are: Ser239, Ser267 (Gly only), His268, Glu293, Gln295, Tyr296, Arg301, Val303, Lys338, Asp376. In the Fc crystal structure, these residues separate into two groups. Lys338 and Asp376 are at the CH2-CH3 interface and may affect the spatial relationship of these two domains, thereby affecting FcγRIIIA binding; note, though, that changing these two residues did not significantly reduce binding to FcγRI, FcγRII or FcRn. The other eight positions are clustered together near the Class 1 residues at the hinge-proximal end of the CH2 domain; of these, only Ser239, Ser267, and His268 were cited as part of the binding site in the Fc:FcγRIIIA crystal structure report (Sondermann *et al.* *EMBO J.* 18: 1095-1103 (1999)). Of the remaining seven, a few might conceivably exert their effect by conformational change, *e.g.* Tyr296, Arg301, Val303, Lys338, and Asp376 (Fig. 26B). However, the Glu293 and Gln295 sidechains are quite solvent exposed, based on Fc crystal structures (Deisenhofer, J. *Biochemistry* 20: 2361-2370 (1981); Guddat *et al.* *Proc. Natl. Acad. Sci. USA* 90:4271-4275 (1993)), and are not involved in interactions which would hint at a conformational role. In addition to Glu293Ala reducing binding by 70% (Class 8), the more conservative change of Glu293Asp also showed a similar reduction (Table 7) implying that Glu293 can indeed interact with FcγRIIIA.

Variants which improved binding to FcγRIIIA (Classes 3, 7 and 9) include Thr256Ala, Lys290Ala, Ser298Ala, Glu333Ala, Lys334Ala and Ala339Thr. Of these, only Ser298 was cited as part of the binding site in the Fc:FcγRIIIA crystal structure report (Sondermann *et al.* *EMBO J.* 18: 1095-1103 (1999)). Though Glu333 and Lys334 do not interact with FcγRIIIA in the co-crystal structure, their interaction with FcγRIIIA is supported by four lines of evidence. First, murine IgG2b Glu333Ala exhibited a modest reduction in binding to murine FcγRII (Lund *et al.* *Mol. Immunol.* 29:53-59 (1992)). Though the same might not occur for murine FcγRIII, this shows that residues distant from the hinge region can influence binding to FcγR. Second, several non-Ala variants at Glu333 and Lys334 either improved or reduced binding to FcγRIIIA (Table 7). Third, binding of Glu333Ala and Lys334Ala to FcγRIIIA-expressing CHO cells improved even more than seen in ELISA-based assays (Table 11). Finally, Lys334Ala exhibited a significant increase in ADCC. This increase in ADCC was additive when Lys334Ala was present with Ser298Ala and was further enhanced when Glu333Ala was present (Fig. 27).

Several residues which influenced binding, albeit modestly, to FcγRIIIA, FcγRIIA and FcγRIIB, are located at the "bottom" of the CH3 domain distant from the larger set of residues in the CH2 domain which exhibited a more pronounced effect on binding. Lys414 (Fig. 26A)

showed a 40% reduction in binding for Fc γ RIIA and Fc γ RIIB (Class 6), Arg416 a 30% reduction for Fc γ RIIA and Fc γ RIIIA, Gln419 a 30% reduction to Fc γ RIIA and a 40% reduction to Fc γ RIIB, and Lys360 a 23% improvement to Fc γ RIIIA (Class 10). Taken together, their effect on binding of IgG1 to Fc γ RIIA, Fc γ RIIB and Fc γ RIIIA suggests that the "bottom" of IgG1 may indeed be involved in the IgG1:Fc γ R interaction, though it may play only a minor role.

Previous studies have mapped the binding site of murine IgG for murine FcRn (Ghetie *et al. Nature Biotech.* 15: 637-640 (1997); Burmeister *et al. Eur. J. Immunol.* 24: 542-548 (1994); Kim *et al. Eur. J. Immunol.* 24: 2429-2434 (1994); Ghetie *et al. Scand. J. Immunol.* 40: 457-465 (1994); Medesan *et al. J. Immunol.* 158: 2211-2217 (1997)). These studies have implicated murine IgG residues Ile253, His310, Gln311, His433, Asn434, His435, and His436 as contacts for one FcRn molecule and Glu272 and His285 as contacts for a second FcRn molecule. In addition, the pH dependence of the IgG:FcRn interaction has been ascribed to His310 and His433 on IgG (as well as His250 and His251 on FcRn) (Raghavan *et al. Biochemistry* 34: 14649-14657(1995)).

In the current study of the human system, a larger number of residues were found which affected binding of IgG1 to human FcRn. Comparison of the human IgG1 sequence with the crystal structure of rat Fc bound to murine FcRn (Burmeister *et al. Nature* 372: 379-383 (1994)) shows that in the human Fc some of these residues could interact directly with human FcRn: Ile253, Ser254, Lys288, Thr307, Gln311, Asn434, and His435. Near the Fc:FcRn interface in the crystal structure but not interacting directly are: Arg255, Thr256, Asp312, Glu380, Glu382, His433, and Tyr436. In the murine system it was found that altering Asn434 to Ala or Gln did not affect binding to murine FcRn (Ghetie and Ward *Immunol. Today* 18:592-598 (1997); Medesan *et al. J. Immunol.* 158: 2211-2217 (1997)). However, in the human system Asn434Ala exhibited the largest improvement in binding seen for any single Ala substitution (Class 10) as well as showing additivity in combination variants (Table 8). Note that while improvement in binding of the variants to FcRn occurred at pH 7.2 as well as at pH 6.0 (Table 8), none of the variants bound well at pH 7.2. Hence, these single or combination variants may be useful in extending the half-life of human IgG1 in therapeutic antibodies, as previously found for murine IgG (Ghetie *et al. Nature Biotech.* 15: 637-640 (1997)), and fulfill the requirement for binding at pH 6.0 and dissociating at pH 7.2.

Selected IgG variants were also tested for their binding to Fc γ R transfected into mammalian cells. The α -chain extracellular portion of human Fc γ RIIIA was transfected into CHO cells using a GPI-link, whereas for human Fc γ RIIB the full-length receptor was transfected into CHO cells. For the variants tested, the pattern of binding to the cells was the same as the pattern of binding in the protein:protein (ELISA) assay (Figs. 18A-B and 19A-B).

Combination Variants

A number of combination variants were tested in which two or more residues were

simultaneously altered to Ala. Some of these combinations showed additive effects. An example is the Glu258Ala/Ser267Ala variant which exhibited binding to FcγRIIA and FcγRIIB that was better than the Glu258Ala (Class 4) and Ser267Ala (Class 4) variants (Tables 6 and 8). A similar outcome was found for the Ser298Ala/Glu333Ala and Ser298Ala/Lys334Ala variants in which the binding to FcγRIIA improved over the parental variants (Table 8). In other combinations, one residue dominated the other, e.g. the Thr256Ala/Ser298Ala variant showed reduced binding to FcγRIIA and FcγRIIB similar to the Ser298Ala variant even though the Thr256Ala change effected better binding to both these receptors (Class 3).

The most pronounced additivity was found for combination variants with improved binding to FcRn. At pH 6.0, the Glu380Ala/Asn343Ala variant showed over 8-fold better binding to FcRn, relative to native IgG1, compared to 2-fold for Glu380Ala and 3.5-fold for Asn434Ala (Tables 6 and 8). Adding Thr307Ala to this effected a 13-fold improvement in binding relative to native IgG1. Likewise, combining Glu380Ala and Leu309Ala, the latter being deleterious to FcRn binding, resulted in a variant which was intermediate between the two parental variants (Table 8). As with the FcγR, some combinations showed dominance of one residue over the other; for the Lys288Ala/Asn434Ala variant, the better binding due to Asn434Ala clearly overcame the reduction in binding from Lys288Ala (Table 8). At pH 7.2 none of variants bound well.

Combining S298(317)A with K334(353)A improved binding to FcγRIIA more than either S298(317)A or K334(353)A alone (Figs. 18A and B; and compare the variants in Tables 6 and 8) (residue numbers in parentheses are those of the EU index as in Kabat). Similarly, combining S298(317)A with E333(352)A improved binding to FcγRIIA more than either S298(317)A or E333(352)A alone (compare the variants in Tables 6 and 8).

ADCC Activity of the Variants

One application of these variants is to improve the ADCC effector function of an antibody. This can be achieved by modifying Fc region amino acids at one or more residues which would lead to improved binding to FcγRIIA. Improved FcγRIIA binding would lead to improved binding by NK cells, which carry only FcγRIIA and can mediate ADCC. Selected alanine variants which were either reduced in binding to FcγRIIA (variants D265(278)A, E269(282)A, D270(283)A, Q295(312)A; Table 6), had no effect on FcγRIIA binding (R292(309)A; Table 6), or had improved binding to FcγRIIA (variants K290(307)A, S298(317)A; Table 6) were tested in an *in vitro* ADCC assay using human PBMCs as effector cells. Since the target cells were HER2-overexpressing SKBR3 cells, the IgG Fc variants used in this assay were generated by substituting the V_H/V_L domains of anti-IgE E27 with those from anti-HER2 antibody; HERCEPTIN® (humAb4D5-8 in Table 1 of Carter *et al.* PNAS (USA) 89:4285-4289 (1992)). The pattern of ADCC exhibited by the variants correlated well with the pattern of binding to FcγRIIA (Figs. 20 and 21). Notably the variant which showed the best improvement in binding to FcγRIIA

in protein:protein assays, variant S298(317)A, also showed improvement in ADCC compared to wildtype HERCEPTIN® at 1.25 ng/ml (Fig. 21).

One set of ADCC assays with PBMCs compared the effect of Asp265Ala (Class 1), Arg292Ala (Class 6), and Ser298Ala (Class 7). The assay was repeated using four different donors. Fig. 24 shows that the ADCC pattern of the variants reiterated that seen in the ELISA binding assay for FcγRIIIA: Asp265Ala prevented ADCC ($P < 0.01$; paired t-test), Arg292Ala had no effect, and Ser298Ala statistically improved ADCC ($P < 0.01$; paired t-test).

A second set of assays were performed in which the FcγRIIIA allotype of the donors were determined. Using three FcγRIIIA-Val158/Val158 and three FcγRIIIA-Phe158/Phe158 donors, ADCC assays using only NK cells were repeated 3-4 times for each donor. Representative ADCC plots are shown in Figs. 27A and 27B and the summary of all assays is shown in Fig. 27C. The variants tested were: Ser298Ala (Class 7), Lys334Ala (Class 9), Ser298Ala/Lys334Ala (Table 8), and Ser298Ala/Glu333Ala/Lys334Ala (Table 8). In agreement with the binding exhibited in the ELISA-format assay (Tables 6, 8 and 11), the pattern of improved ADCC was Ser298Ala/Glu333Ala/Lys334Ala > Ser298Ala/Lys334Ala > Ser298Ala = Lys334Ala. This pattern was seen with both the FcγRIIIA-Phe158/Phe158 and FcγRIIIA-Val158/Val158 donors, though improvement in ADCC was less pronounced for the latter. Comparing the improvement in binding to receptor for these variants in the ELISA-format assay (Tables 6, 8 and 11) with that in the cell-based (Table 11) and ADCC assays (Figs. 24 and 27) shows that the improvement in binding for any specific variant is enhanced when the receptor is expressed on cells.

EXAMPLE 5

Bind of Fc Variants to Polymorphic Fc Receptors

Allelic variants of several of the human FcγR have been found in the human population. These allelic variant forms have been shown to exhibit differences in binding of human and murine IgG and a number of association studies have correlated clinical outcomes with the presence of specific allelic forms (reviewed in Lehrnbecher *et al. Blood* 94(12):4220-4232 (1999)). Several studies have investigated two forms of FcγRIIA, R131 and H131, and their association with clinical outcomes (Hatta *et al. Genes and Immunity* 1:53-60 (1999); Yap *et al. Lupus* 8:305-310 (1999); and Lorenz *et al. European J. Immunogenetics* 22:397-401 (1995)). Two allelic forms of FcγRIIIA, F158 and V158, are only now being investigated (Lehrnbecher *et al., supra*; and Wu *et al. J. Clin. Invest.* 100(5):1059-1070 (1997)). In this example, selected IgG variants were tested against both allelic forms of FcγRIIA or FcγRIIIA. Fc receptor binding assays were performed essentially as described in the above examples. However, for FcγRIIIA-V158, both (a) the low affinity receptor binding assay of Example 1 (which analyzes binding of the IgG complex to FcγRIIIA-V158); and (b) the high affinity FcγR binding assay of Example 4

(which analyzes binding of IgG monomer to FcγRIIIA-V158) were carried out. The CHO stable cell lines expressing FcγRIIIA-Phe158 and FcγRIIIA-Val158 with human γ-chain were generated by subcloning the α-chain and γ-chains into a previously described vector which includes DNA encoding a green fluorescent protein (GFP) (Lucas *et al. Nucl. Acids Res.* 24: 1774-1779 (1996)). CHO cell transfection was carried out using Superfect (Qiagen) according to the manufacturer's instructions. FACS sorting was done based on GFP expression as described previously (Meng *et al. Gene* 242: 201-207 (2000)). Receptor expression levels were determined by staining with anti-FcγRIII monoclonal antibody 3G8 (Medarex). Binding of IgG1 variants was performed by adding monomeric IgG in staining buffer to 5×10^5 cells and incubating in 96 well round-bottom tissue culture plates (Costar, Cambridge, MA) at 4°C for 30 min. Cells were washed three times with staining buffer and IgG binding detected by addition of 1:200 PE-F(ab')₂ fragment of goat anti-human IgG and incubation for 30 min at 4°C. After washing, immunofluorescence staining was analyzed on a FACScan flow cytometer using Cellquest software (Becton Dickinson). Dead cells were excluded from analysis by addition of 1 μg/ml propidium iodide. The results of these studies are summarized in Table 9-11 below.

TABLE 9
Binding of Variants to FcγRIIA and FcγRIIIA Polymorphic Receptors

	Res#EJ (Kabat)	IgG Complex			FcγRIIA-H131			IgG Complex			FcγRIIIA-V158			IgG Monomer		
		mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n	mean	sd	n
5	IG2															
10	11	T25E(269)A	1.41	(0.27)	9			1.22	(0.18)	9	0.97	(0.03)	2	1.20		1
	254	T25E(269)N	1.03		1			1.13		1	0.95		1	0.88		1
	14	D265(278)A	0.07	(0.01)	4			0.09	(0.06)	4	0.01		1			
15	15	S267(280)A	1.64	(0.18)	7	1.05	(0.03)	2	1.14	(0.25)	7					
	189	S267(280)G	1.21	(0.05)	3	0.59	(0.09)	3	0.09	(0.02)	3					
	16	H268(281)A	1.22	(0.14)	12	1.09	(0.01)	2	0.52	(0.02)	12					
20	25	E283(300)A	1.24	(0.23)	5			1.01	(0.14)	5				0.78		1
	226	E283(300)Q	1.12		1			1.19		1				0.89		1
	227	E283(300)S	1.03		1			0.85		1				0.83		1
25	228	E283(300)N	1.18		1			0.94		1				0.63		1
	229	E283(300)D	1.14		1			0.95		1				0.67		1
30	30	K290(307)A	1.29	(0.21)	7			1.28	(0.21)	7	1.12	(0.05)	2	1.13		1
	73	K290(307)Q	1.17		1			1.40		1	1.02		1	1.30		1
	75	K290(307)S	1.27		1			1.26		1	1.05		1	1.62		1
35	77	K290(307)E	1.10		1			1.30		1	0.98		1	1.50		1
	78	K290(307)R	1.05		1			1.08		1	1.07		1	1.24		1
40	177	K290(307)G	1.07		1			1.23		1	1.11		1	2.29		1
	31	P292(309)A	0.27	(0.14)	9			0.90	(0.18)	9				0.94		1
	80	R292(309)K	0.71	(0.17)	3			1.15	(0.18)	3				1.64		1

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5	81	R292(309)H	0.21 (0.09) 2	0.92 (0.09) 2	1.21	1
	82	R292(309)Q	0.47 (0.12) 3	0.45 (0.09) 3	0.56	1
	83	R292(309)N	0.54 (0.16) 3	0.88 (0.02) 3	0.91	1
10	144	E293(310)Q	0.85 (0.03) 2	0.99 (0.04) 2	1.00	1
	33	E294(311)A	0.87 (0.19) 5	0.66 (0.14) 5	0.68	1
	173	E294(311)Q	1.01	0.84	0.79	1
15	174	E294(311)D	0.37	0.14	0.26	1
	36	S298(317)A	0.40 (0.08) 12	1.30 (0.18) 12	1.02 (0.04) 2	1
	70	S298(317)G	0.87 (0.17) 4	0.46 (0.09) 4	0.88	1
20	71	S298(317)T	0.41 (0.21) 3	0.89 (0.20) 3	0.96	1
	72	S298(317)N	0.08 (0.01) 2	0.06 (0.01) 2	0.66	1
	218	S298(317)V	0.11 (0.06) 3	0.33 (0.19) 3	0.88	1
25	219	S298(317)L	1.14 (0.12) 3	0.34 (0.04) 3	0.83	1
	40	V305(324)A	1.12 (0.12) 4	1.04	0.84 (0.15) 4	
	41	T307(326)A	1.19 (0.37) 12	1.37 (0.13) 2	1.12 (0.18) 12	
30	45	N315(334)A	1.15 (0.06) 5	1.11 (0.06) 2	1.07 (0.21) 5	
	46	K317(336)A	1.13 (0.05) 4	1.04	1.10 (0.23) 4	
	48	K320(339)A	1.12 (0.11) 4	1.16	0.87 (0.17) 4	
35	54	E333(352)A	0.92 (0.12) 10	1.27 (0.17) 10	1.10 (0.10) 2	1
	141	E333(352)Q	0.70 (0.05) 2	1.10 (0.03) 2	1.05	1
	142	E333(352)N	0.59 (0.04) 2	0.56 (0.10) 2	0.64	1
40	143	E333(352)S		0.94	0.99	1
	45					

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152	E333(352)K		0.85 (0.14)	3	0.88	1	0.81	1
153	E333(352)E	0.75 (0.04)	2	0.84 (0.05)	2	0.92	1	0.83
154	E333(352)D			1.26 (0.04)	3	1.00	1	1.70
178	E333(352)G	0.87	1	1.05	1		1.23	1
55	K334(353)A	1.01 (0.15)	17	1.39 (0.19)	17	1.07 (0.09)	3	1.60 (0.01)
135	K334(353)R	1.15 (0.09)	5	0.68 (0.07)	5		0.88	1
136	K334(353)Q	1.08 (0.11)	7	1.31 (0.26)	7	1.27 (0.01)	2	1.92
137	K334(353)N	1.16 (0.11)	7	1.15 (0.16)	7	1.19 (0.06)	2	1.70
138	K334(353)S	1.01 (0.11)	3	1.19 (0.08)	3	1.25	1	1.82
139	K334(353)E	0.74 (0.15)	4	1.30 (0.09)	4	1.17	1	2.75
140	K334(353)D	0.51 (0.09)	4	1.13 (0.09)	4	1.07	1	
179	K334(352)G	0.76 (0.08)	5	0.88 (0.22)	5	0.94	1	1.28
190	K334(353)M	1.06	1	1.35	1	0.99	1	2.08
191	K334(353)Y	1.08	1	1.31	1	0.98	1	1.72
192	K334(353)W	0.94	1	1.07	1	0.96	1	1.53
193	K334(353)H	1.09	1	1.26	1	0.97	1	2.06
220	K334(353)V	1.13 (0.11)	3	1.34 (0.18)	3	1.00	1	2.89
221	K334(352)L	1.05	1	1.38	1	0.96	1	3.59
65	P331(350)A	1.29 (0.14)	3	1.03 (0.19)	3	0.96	1	0.78
198	P331(350)S	1.00	1	0.86	1		0.54	1
199	P331(350)N	0.86	1	0.23	1		0.24	1
200	P331(350)E	1.06	1	0.42	1		0.36	1
203	P331(350)K	0.94	1	0.33	1		0.26	1

96	S267(29C)A H268(281)A	1.54 (0.12)	3	1.07 (0.06)	2	0.84	1	
5	110 S298(317)A E333(352)A K334(353)A	0.35 (0.13)	11	1.66 (0.42)	11	1.19 (0.18)	3	
10	271 E380(405)A L309(328)A	0.98	1	0.92	1	1.10	1	

Table 10
Binding of Human IgG1 Variants to
Human FcγRIIA-R131 and FcγRIIA-H131 Polymorphic Receptors

Variant ^a	Class ^b	FcγR	FcγRIIA-P131 ^c mean (sd) N	FcγRIIA-R131 ^d mean (sd)	FcγRIIA-H131 mean (sd)	H131/R131 mean (sd) N
Ser267Ala	4	!II	1.52 (0.22) 11	1.53 (0.06)	1.10 (0.12)	0.71 (0.07) 5
Ser267Gly		!III	1.18 (0.10) 4		0.54 (0.14)	0.47 (0.13) 5
His269Ala	5	!II !III	1.21 (0.14) 12	1.30 (0.17)	0.97 (0.15)	0.75 (0.12) 10
Asp270Ala	2	!II, !III	0.06 (0.01) 5	0.04 (0.02)	0.45 (0.11)	16.6 (8.5) 6
Ser298Ala	7	!II !III	0.40 (0.15) 16	0.26 (0.10)	0.24 (0.08)	0.93 (0.13) 6
Val305Ala	10	!FcRn	1.12 (0.12) 4	1.00 (0.14)	1.06 (0.10)	1.08 (0.10) 4
Thr307Ala	4	!II	1.07 (0.14) 11	1.28 (0.13)	1.18 (0.06)	0.94 (0.09) 5
Asn315Ala	4	!II	1.15 (0.06) 5	1.11 (0.18)	1.10 (0.16)	0.99 (0.05) 8
Lys317Ala	10	!FcRn	1.13 (0.05) 4	1.10 (0.13)	1.08 (0.08)	0.99 (0.07) 7
Lys320Ala		no effect	1.14 (0.11) 6	1.05 (0.19)	1.13 (0.09)	1.10 (0.15) 7

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Ser267Ala III 1.41 (0.00) 2 1.57 (0.06) 1.02 (0.08) 0.65 (0.03) 4
His268Ala

a Residue numbers are according to the Eu numbering system (Kabat et al. (1991) supra).

b Class as noted in Table 6.

c Values are from Table 6, 7 or 8.

d Values are the ratio of binding of the variant to that of native IgG1 in assays separate from those in column 4 and performed simultaneously with the FcγRIIIA-H131 assays in column 6.

Table 11

Binding of Human IgG1 Variants to Human FcγRIIIA-Phe158 and FcγRIIIA-Val158 Polymorphic Receptors

Variant ^a	Class ^b	FcγR	Phe158 ^c mean (sd)	N	Phe158 ^d mean (sd)	Val158 ^e mean (sd)	N ^f
Asp265Ala	1	I,II,III	0.09 (0.06)	4	0.05 (0.02) 0.12 (0.08)	0.02 (0.01) 0.05 (0.02)	5 3
Lys290Ala	3	I,II,III	1.31 (0.19)	9	1.15 (0.27) 1.61 (0.15)	1.01 (0.08) 0.89 (0.04)	4 3
Ser298Ala	7	I,II,III	1.34 (0.20)	16	1.49 (0.27) 1.85 (0.05)	1.07 (0.07) 1.18 (0.09)	7 3
Pro331Ala	4	I,II	1.08 (0.19)	3	1.00 (0.23) 0.94 (0.07)	0.97 (0.02) 0.88 (0.09)	5 3
Glu333Ala	9	I,II,III	1.27 (0.17)	10	1.13 (0.32) 1.42 (0.04)	1.06 (0.11) 1.08 (0.09)	4 3
Lys334Ala	9	I,II,III	1.39 (0.19)	17	1.39 (0.22) 2.46 (0.08)	1.10 (0.07) 1.26 (0.21)	9 3
Ser298Ala		I,II,III	1.51 (0.31)	10	2.17 (0.36) 3.42 (0.28)	1.11 (0.08) 1.65 (0.12)	5 3

a Residue numbers are according to the Eu numbering system (Kabat et al. (1991) supra).

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- b Class as noted in Table 6.
 - c Values are for FcγRIIIA-Phel58 from Table 6.
 - d Values are the ratio of binding of the variant to that of native IgG1 to FcγRIIIA-Phel58 in assays separate from those in column 4 and performed simultaneously with the FcγRIIIA-Vall58 assays in column 6. Upper values are for binding in the ELISA format assay; lower values are for binding to CHO cells stable transfected with the α- and γ- chains of the receptor.
 - e Values are the ratio of binding of the variant to that of native IgG1 to FcγRIIIA-Vl58. Upper values are for binding in the ELISA format assay; lower values are for binding to CHO cells stable transfected with the α- and γ- chains of the receptor.
 - f Number of independent assays for values in columns 5 and 6.
- 5
- 10

Selected variants were tested for binding to the Fc γ RIIA-H131 and Fc γ RIIA-V158 allotypic receptor forms based on their improved or reduced binding to the allotypic forms used for the assays (*i.e.* Fc γ RIIA-Arg131 and Fc γ RIIA-Phe158). Tables 9 and 10 show that most of the variants bound equivalently to the Fc γ RIIA-Arg131 and Fc γ RIIA-His131 receptors. The exceptions were the Ser267Ala, His268Ala and Ser267Ala/His268Ala variants which displayed binding to Fc γ RIIA-His131 that was reduced compared to Fc γ RIIA-Arg131 but still equivalent to native IgG1. The related Ser267Gly variant, however, showed a 50% reduction in binding to the Fc γ RIIA-His131 receptor compared to native IgG1. In contrast to Ser267Ala and His268Ala, Asp270Ala reduced binding to Fc γ RIIA-His131 by 50% but completely abrogating binding to Fc γ RIIA-Arg131. This suggests that Ser267, His268 and Asp270 interact with Fc γ RIIA in the vicinity of Fc γ RIIA residue 131.

For Fc γ RIIA, the selected variants were assayed in the ELISA-format as well as on stable-transfected CHO cell lines expressing the γ -chains (Fc γ RIIA-Phe158 or Fc γ RIIA-Val158) with the associated human α -chain. For Fc γ RIIA-Phe158, those variants that showed improved binding in the ELISA-format exhibited even more improvement, compared to native IgG1, in the cell-based assay (Table 11). This could be due to the presence of the γ -chain associated with the α -chain enhancing binding of the IgG to Fc γ RIIA (Miller *et al. J. Exp. Med.* 183:2227-2233 (1996)). Alternatively, since the cell-based assay utilized monomeric IgG (in contrast to hexameric complexes used in the ELISA-format assay), the cell-based assay may be less subject to an avidity component and thus more sensitive to changes in the binding interface. In contrast, none of the variants exhibited improved binding to the Fc γ RIIA-Val158 receptor in the ELISA-format assay, though the Ser298Ala, Lys334Ala and Ser298Ala/Glu333Ala/Lys334Ala variants did bind better than native IgG1 in the cell-based assay.

Human Fc γ RIIA has two known, naturally occurring allotypes which are determined by the amino acid at position 131. Among the human IgG1 variants tested against both Fc γ RIIA-Arg131 and Fc γ RIIA-His131, variants Ser267Ala, Ser267Gly, His268Ala and Asp270Ala could discriminate between the polymorphic forms. This suggests that these IgG1 residues interact with Fc γ RIIA in the vicinity of Fc γ RIIA residue 131 and in the IgG1 Fc:Fc γ RIIA crystal structure (Sondermann *et al. Nature* 406:267-273 (2000)), Ser267 is adjacent to His131.

Human Fc γ RIIA has naturally occurring allotypes at position 48 (Leu, His or Arg) and at position 158 (Val or Phe). The Fc γ RIIA-Val158 allotype binds human IgG better than the Fc γ RIIA-Phe158 allotype (Koene *et al. Blood* 90: 1109-1114 (1997); and Wu *et al. J. Clin. Invest.* 100: 1059-1070 (1997)) and this difference is reiterated in the ELISA-format, cell-based

and ADCC assays in this study. The IgG1 Fc:FcγRIIIA crystal structure offers an explanation for this difference. In the crystal structure, V158 interacts with the IgG1 lower hinge near Leu235-Gly236 and with the FcγRIIIA Trp87 sidechain (which in turn interacts with the important IgG1 Pro329); introduction of the larger Phe158 may alter either or both of these interactions and thereby reduce the binding.

Some of the IgG1 variants exhibited better binding to FcγRIIIA-Phe158 (e.g. Classes 7 and 9) and could be further improved by combining individual variants (Table 8). These same variants showed no or minimal improvement in binding to FcγRIIIA-Val158 in the ELISA-format assay. However, when tested on cells expressing FcγRIIIA-Val158 or in ADCC assays using FcγRIIIA-Val158/Val158 donors, some of these variants did show superior interaction compared to native IgG1 (Table 11, Fig. 27). Comparing the ADCC results of select IgG1 variants with better binding to FcγRIIIA, the variants exhibited a significant improvement in ADCC for both FcγRIIIA-Phe158/Phe158 and FcγRIIIA-Val158/Val158 donors (Fig. 27). Indeed, using the Ser298Ala/Glu333Ala/Lys334Ala variant, the FcγRIIIA-Phe158/Phe158 donor ADCC could be increased over 100% (i.e. > 2-fold) compared to native IgG1 (Fig. 27C).

While the influence of FcγRIIIA polymorphic forms in various human diseases has been investigated for many years (reviewed in Lehnbecher *et al. Blood* 94:4220-4232 (1999)), the possible correlation between FcγRIIIA polymorphic forms and human disease has only recently been investigated (Wu *et al. J. Clin. Invest.* 100: 1059-1070 (1997); Lehnbecher *et al. Blood* 95: 2386-2390 (2000); Nieto *et al. Arthritis & Rheumatism* 43: 735-739 (2000)). Given the possible involvement of FcγR in the mechanism of action of therapeutic antibodies, human IgG1 variants with improved binding capacity to human FcγR, especially variants with better binding to FcγRIIIA and simultaneous abrogation of binding to the inhibitory FcγRIIB, could be used to provide more efficacious therapeutic antibodies. In addition, a recent report on the occurrence of polymorphic FcγR forms in control populations showed that the FcγRIIIA-Phe158 allele is more prevalent than the FcγRIIIA-Val158 allele (Lehnbecher *et al. Blood* 94: 4220-4232 (1999)). Since the FcγRIIIA-Phe158 receptor binds human IgG1 less well than the FcγRIIIA-Val158 receptor, therapeutic antibodies with variant Fc portions that improve binding to FcγRIIIA-Phe158 at least to the level seen for FcγRIIIA-Val158 (if not more so) could provide increased therapeutic efficacy to the majority of the population.

Example 6

Role of IgG Residues Affecting Carbohydrate

To determine whether differences in binding among variants was related to variation in the oligosaccharide at the conserved Asn297-linked glycosylation site, oligosaccharides of

various IgG variants were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) as previously described (Papac *et al. Glycobiology* 8: 445-454 (1998)). Following immobilization of approximately 50 µg of IgG to PVDF membranes in 96 well MultiScreen® IP plates (Millipore), proteins were reduced with 50 µL 0.1 M dithiothreitol in 8 M urea, 360 mM Tris, 3.2 mM EDTA, pH 8.6 (RCM buffer). Resultant free sulfhydryl groups were subsequently carboxymethylated by incubation with 0.1 M iodoacetic acid in RCM buffer at 25°C for 30 min in the dark. Prior to enzymatic release of glycoproteins, membrane-bound proteins were incubated in 1% aqueous polyvinylpyrrolidone 360 (Sigma) solution at 25°C for 1 hr. Oligosaccharides were released by incubating protein with 32 units of PNGase F (New England Biolabs, Beverly, MA) in 25 µL of Tris-acetate buffer, pH 8.4, at 37°C for 3 hr, followed by acidification by addition of 2.5 µL 1.5 M acetic acid and then incubated for 25°C for 3 hr. Samples were then purified by cation exchange chromatography using hydrogen form, 100-200 mesh AG50W-X8 resin (Biorad). Released oligosaccharides were analyzed by MALDI-TOF-MS in both positive and negative modes using matrices containing 2,5-dihydroxybenzoic acid (DHB) and 2,4,6-trihydroxyacetophenone (THAP), respectively (Papac *et al. Anal. Chem.* 68: 3215-3223 (1996)). Analysis was performed on a Voyager DE mass spectrometer (PerSeptive Biosystems, Foster City, CA) by transferring 0.5 µL of sample to a stainless steel target containing 0.4 µL of the appropriate matrix. Following vacuum dessication, the samples were ionized by irradiation with an N2 laser (337 nm wavelength) and ions were accelerated with a 20 kV voltage. Ion mass assignment was made using oligosaccharide standards (Oxford Glycosciences, Rosedale, NY) in a two-point external calibration. Final spectra were the result of the summation of the individual spectral data from 240 laser ignitions. The results are shown in Table 12 below.

Table 12
Percent of Total Oligosaccharide Area by Glycan Type

		IgG1	Glu258 Ala	Asp265 Ala	Tyr296 Phe	Ser298 Ala	Arg301 Ala	Arg301 Met	Val303 Ala	Lys334 Ala
5										
		=====								
		High Mannose								
		14.6 ^a	11.1	8.0	14.8	11.3	5.3	5.4	10.2	20.9
		3.8	1.1	4.0			0.8	2.4	0.7	
10		=====								
		Complexes with Terminal Galactose								
	0	51.5	36.3	47.2	47.0	48.9	24.1	24.3	48.7	49.5
		0.6	0.2	6.0			3.7	0.9	0.2	
15	1	23.4	34.9	25.0	23.1	28.1	23.9	27.2	28.5	17.4
		3.2	0.9	3.5			0.8	5.9	0.5	
	2	8.5	15.8	19.5	12.5	9.6	45.3	42.0	11.4	10.8
		0.3	0.1	6.3			2.4	5.9	0.4	
20	3	2.0	2.0	0.2	2.5	2.1	1.1	1.1	1.0	1.5
		0.5	0.2	0.2			1.0	0.3	0.1	
	1-3	-----	-----	-----	-----	-----	-----	-----	-----	-----
25		33.9	52.7	44.7	38.1	39.8	70.3	70.3	40.9	29.7
		4.0	1.2	10.0				4.0	1.7	1.0
		Complexes with Terminal Sialic Acid								
		0.0	3.0	2.4	0.0	0.0	9.7	10.9	1.3	0.0
30		Complexes with Fucose								
	0	4.5	1.1	1.3	3.0	1.5	0.7	1.5	1.6	6.1
		0.6	0.1	0.2			0.2	1.9	0.2	
	1	81.1	87.9	90.7	82.1	87.2	94.0	93.4	88.1	73.1
35		3.0	1.3	3.9			1.0	1.5	0.5	
		Triantennary Complexes								
		0.2	0.2	3.2	0.0	0.0	5.5	5.0	0.3	0.0
		0.2	0.2	0.1			1.2	0.8	0.3	
40		=====								

a Upper values are mean percent and lower values are deviation from mean for two independent analyses on two different lots of IgG.

Previously it was noted that replacing human IgG3 residues which contact the oligosaccharide, e.g. Asp265, Tyr296, Arg301, with Ala resulted in increased galactosylation and sialylation relative to native IgG3 and in reduced binding to both FcγR and C1q (Lund *et al. J. Immunol.* 157: 4963-4969 (1996)). In order to determine if the effect seen for specific Ala substitutions (either deleterious or advantageous) was due to differences in glycosylation, oligosaccharide analysis was performed for selected variants (Table 12). The Asp265Ala,

Arg301Ala and Arg301Met variants showed increased galactosylation, a relatively small amount of sialylation, and a small percentage of triantennary carbohydrate, in agreement with Lund *et al.*, *supra*. The Arg301Ala and Arg301Met variants also showed an increase in fucose and a decrease in mannose not seen previously. For the Tyr296Phe variant, there were no differences from native IgG1, in contrast to the decrease in galactose and fucose and increase in mannose reported by Lund *et al.*, *supra*. These differences may be due to the different mammalian cells used to express the antibodies (human kidney 293 cells in this study and Chinese hamster ovary cells in the previous study) or may reflect that Tyr296 was changed to Phe296 in this study whereas it was changed to Ala296 in the Lund *et al.* study.

The Lys334 sidechain is near the carbohydrate in IgG crystal structures but does not interact with it as intimately as do Asp265, Tyr296 and Arg301. The Lys334Ala variant exhibited a small increase in mannose and small decrease in fucose compared to native IgG1 (Table 12).

Ser298 interacts with the carbohydrate only through its O γ atom, which forms a hydrogen bond to the Asn297 O γ , and no difference in carbohydrate for the Ser298Ala was evident compared to native IgG1. Neither the Glu258 or Val303 sidechains interact with the carbohydrate, indeed both are located on the opposite face of the CH2 domain from the carbohydrate. However, the Glu258Ala variant showed an increase in galactosylation and a small amount of sialic acid while the Val303Ala variant only showed a small amount of sialic acid. Hence, variation in galactosylation and sialic acid for a given variant (compared to native IgG1) may occur regardless of whether the amino acid sidechain interacts with the carbohydrate.

For the Tyr296Phe, Ser298Ala, Val303Ala and Lys334Ala variants the differences in glycosylation, compared to native IgG1, were minimal and most likely were not the cause of the differences in binding of these variants to the Fc γ R. For the Glu258Ala, Asp265Ala, Arg301Ala and Arg301Met variants, it is difficult to discern whether reduction or improvement in Fc γ R binding is due to the change in amino acid sidechain or from differences in glycosylation.

The presence of carbohydrate linked at residue Asn297 is required for binding to Fc γ R (Lund *et al.*, *supra*). In addition, the nature of the carbohydrate can influence binding (Umaña *et al. Nature Biotech.* 17: 176-180 (1999); Lifely *et al. Glycobiology* 5 :813-822 (1995) ; Lund *et al. J. Immunol.* 157: 4963-4969 (1996); and Lund *et al. FASEB J.* 9: 115-119 (1995)). In crystal structures of IgG (Fc and intact antibody), Asp265 interacts directly with the Asn297-linked carbohydrate via hydrogen bonds (Deisenhofer, J. *Biochemistry* 20: 2361-2370 (1981); Guddat *et al. Proc. Natl. Acad. Sci. USA* 90: 4271-4275 (1993); Harris *et al. J. Mol. Biol.* 275: 861-872 (1998); and Harris *et al. Biochemistry* 36: 1581-1597 (1997)). Previous studies found that an Asp265Ala change in human IgG3 altered the composition of the Asn297-linked carbohydrate and reduced binding to Fc γ RI (Lund *et al. J. Immunol.* 157: 4963-4969 (1996); and Lund *et al.*

FASEB J. 9: 115-119 (1995)). In human IgG1, Asp265Ala (Class 1) the carbohydrate also differed from that of native IgG1 (Table 12) and binding to FcγRI was reduced. Variants at position 258 and 301 also showed variation from native IgG1 and the other variants (Table 12). The two Arg301 variants exhibited an increase in binding to FcγRIIB, a decrease in binding to FcγRIIIA, and no effect on binding to FcγRI or FcRn (Class 5). Asp265Ala (Class 1) showed decreased binding to all FcγR while Glu258Ala (Class 4) showed increased binding to FcγRII only. Hence, while it is possible that the idiosyncratic carbohydrate on these variants influenced binding rather than the amino acid changes directly affecting interaction with the FcγR, the data do not allow resolution of the two possibilities. For the Tyr296Phe, Ser298Ala, Val303Ala and Lys334Ala variants there were no significant differences in carbohydrate from that of native IgG1 (Table 12). Hence the differences in binding to the various FcγR exhibited by these variants is unlikely to be a result of glycosylation differences.